

# PROGRESS IN BOTANY 59

Edited by H.-D. Behnke  
K. Esser  
J. W. Kadereit  
U. Lüttge  
M. Runge

Genetics  
Cell Biology and Physiology  
Ecology and  
Vegetation Science



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Edited by

H.-D. Behnke, Heidelberg  
K. Esser, Bochum  
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## Editorial

Dear Readers,

In this new volume of "Progress in Botany" you will find a number of changes that go back to suggestions we have received over the last few years. In order to adapt the series to the developments in our field, we thought it justified to alter the series' concept accordingly.

The most obvious change concerns the visual presentation of the series; we hope that you will like the new design starting with this volume.

The other major change concerns the organisation of the contents of this and future volumes. "Special Topics" is no longer included and the sections of each volume have been re-arranged into "Genetics", "Cell Biology and Physiology", "Systematics and Comparative Morphology", and "Ecology and Vegetation Science". However, updates for each section will not necessarily occur in every volume; some sections may have contributions only every other year, depending on the "progress" in the respective subfield since the release of the previous volume. According to the unchanged philosophy of the „Progress" series, we do not strive for "completeness". Our goal is to report only true progress. Furthermore, taking into consideration that all areas of the plant sciences show increasing overlap, it was decided that not just one editor should be in charge of one particular section, appearing under his name, but that all of us should share the responsibility for all sections. Consequently, you will not find our names with the section headings but following each individual contribution.

Another often formulated request from our readers who would like to obtain the original publications concerns the citation of literature in "Progress". Somewhat reluctantly – due to the resulting increase in the size of the already large volumes – we, together with the publisher, have decided that all literature citations should be given in a more comprehensive form, that is, with the full titles of the cited publications.

For more than 60 years, "Progress in Botany" has successfully maintained its high standard, and we are confident of achieving our goals also in the future. To ensure this, we invite you – the readers – to continue giving us your criticism and suggestions.

The Editors and Springer-Verlag



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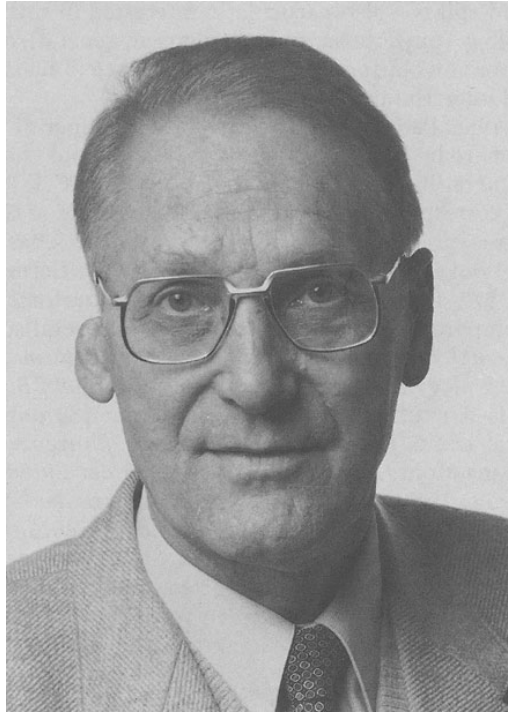
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**Peter Sitte** was born in 1929 at Innsbruck, Austria, and studied biology, chemistry, physics and philosophy at the university of his home town where he received his Ph.D. in 1954. Already in his doctoral thesis on the fine structure of plant cell walls he became involved, together with his elder brother, Hellmuth Sitte (who constructed the famous "Ultracut" ultramicrotome), in the then rapidly developing biological applications of electron microscopy. He habilitated in 1958 at Innsbruck University for General Biology. Immediately after, he moved to Heidelberg University where he soon became associate professor of cytology and biological electron microscopy at the Botanical Institute. Whilst still in Austria, he married Dr. Eva von Lürzer. They have two children.

After having given temporary guest lectures and courses in Stockholm, Darmstadt, Austin (Texas) and Salzburg, he became full professor of cell biology at Freiburg University in 1966 where he worked and taught until his retirement in 1995 (and, on a reduced scale, also since then). His main interests have been focused on cell fine structure, electron and polarizing microscopy and in particular on suberized cell walls, chromoplasts, cytosymbiotic systems and the evolution of complex cells and the secondary plastids of the cryptomonads. Furthermore, Peter

Sitte was – and still is – also particularly interested in certain problems of biomorphology (phyllotaxis, and symmetry in general), of inheritance (genetic information beyond the one stored in nucleic acids), the history of science and interrelations of science and art.

Already in 1968, Peter Sitte was elected as a member of the Academy Leopoldina, where he was a member of the senate and chairman of the sections of General Biology (1973–1981) and later of Cell Biology (since 1991). He is a corresponding member at the academies at Göttingen and Vienna. He was president of diverse scientific societies (1973–1975, German Society of Electron Microscopy; 1975–1977, German Society of Cell Biology; 1977–1978, Gesellschaft Deutscher Naturforscher und Ärzte). He was temporarily engaged as (co)editor of several scientific journals (*Z. Pflanzenphysiol.*, *Fortschr. d. Botanik*, *Protoplasma*, *Grana palynologica*, *Cell Biology Monographs*, *Europ. J. Cell Biol.*, *Biologie in unserer Zeit*). His scientific oeuvre comprises about 180 publications, including several books (*Bau und Feinbau der Pflanzenzelle*, 1965; together with Hans Mohr: *Molekulare Grundlagen der Entwicklung*, 1971; together with G.C. Hirsch and H. Ruska: *Grundlagen der Cytologie*, 1973 and 1974; together with Hans Kleinig: *Zellbiologie – ein Lehrbuch*, 1984, 1986, 1992). Recently, he revised the first part of Strasburger's renowned *Lehrbuch der Botanik für Hochschulen* (33 ed., 1991).

Peter Sitte was awarded the Schleiden medal (Leopoldina, 1991), the Lorenz Oken medal (1992) and the honorary title Dr. rer. nat. h.c. at the University of Salzburg (1995).



## Facts and Concepts in Cell Compartmentation

By Peter Sitte

*"Eukaryotic life depends on the spatial and temporal organization of cellular membrane systems" (Rothman and Wieland 1996).*

### 1. Introduction

The discovery of intracellular membrane systems in the earliest days of biomedical electron microscopy has since proven to be of prime importance for cell biology and biochemistry. Endomembranes completely enclose diverse compartments in eukaryotic cells (eucytes) that, due to their particular respective complements of enzymes, carry out variable metabolic functions.

The various kinds of endomembranes are distinctively different in their lipid and protein composition, particularly in their complement of ion channels and translocators (Heldt and Flüggé 1992; Chrispeels et al. 1995). A unique metabolic milieu can be maintained in any particular compartment only by specific and active membrane transport systems (cf. Tobin 1992). Membrane potentials and concentration gradients at compartment borders can be used for energy conversion and conversation. External signals are received at membranes and transduced into the compartments (cf. Bowler and Chua 1994; Schroeder 1995; Zentgraf and Hemleben 1996; Barnes et al. 1997). Nevertheless, biomembranes act primarily as barriers against free diffusion. Otherwise, any concentration gradients between cellular compartments would be abolished within seconds due to the (sub)microscopic dimensions of the compartments. Consequently, ionophores, digitonin, polyene antibiotics, complement, and other factors which "puncture" membranes kill cells by eliminating membrane potentials (cf. Bhakdi 1988).

Although many cellular processes are not governed by membranes but by direct interactions of different components, particularly by enzyme complexes leading to metabolic channeling, the internal compartmentation of eucytes opens some additional possibilities that have been amply exploited in evolution:

- Membrane-connected reactions such as, e.g., electron transport chains can be maintained at an adequate intensity even in enlarged cells. "I think that there is ... a correlation between an increase in size and an increase in the division of labour, and that this is a fact of tremendous significance" (Bonner 1995).

- Membrane potentials can be built up not only at the plasma membrane but also inside the cell, where they may be used for energy conservation or for energizing active transport.
- Despite the lateral mobility of membrane lipids and proteins (cf. Storrie and Kreis 1996), membrane differentiation becomes possible by membrane separation.
- Within a given cell, domains with different pH, ion content, and redox conditions, together with a given concentration of particular enzymes and metabolites as well as isolation from disturbing influences, allow for efficient metabolic activities. For this purpose, the membranes of compartments must be permeable for metabolites at the starting points of reaction chains or cycles as well as for the respective end products. They have to be, however, impermeable for intermediates.
- The enzyme complement responsible for special functions of a given compartment can be established by regulated protein import (protein kinesis).
- Bulk transport of the content of compartments can take place by vesicular traffic, without permeation across membranes. This permits the shifting of particles of even microscopic dimension such as wall scales in some algae, or mastigonemes in many flagellates for which translocators do not (and apparently cannot) exist.
- Vesicle transport typically proceeds only in one direction, contrary to open connections between compartments which would allow transport back and forth.

**Table 1.** Compartments in plant cells

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Plasmatic compartments<sup>a</sup>:

Cytoplasm ("cytosol")  
 Nucleoplasm ("karyoplasm")  
 Plastid stroma ("plastoplasm")  
 Mitochondrial matrix ("mitoplasm")

Exoplasmatic compartments<sup>a</sup>:

Vacuoles  
 Microbodies (peroxisomes)<sup>b</sup>  
 RER (rough ER, including NE)  
 SER (smooth ER)  
 Golgi cisternae  
 Vesicles of different kind (e.g., Golgi cisternae, CVs)  
 Intermembrane space of mitochondrial envelope  
 Intermembrane space of plastid envelope  
 Chloroplast thylakoids

Storage lipid compartments:

Oleosomes

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<sup>a</sup> See Section 12.

<sup>b</sup> On a possible "plasmatic" nature of peroxisomes see Section 11.

**Table 2.** Volume proportions of compartments in plant cells (in % of protoplast volume)

	Embryonic cells <sup>a</sup>	Mesophyll cells Barley <sup>b</sup>	Spinach <sup>c</sup>
Cytoplasm	41.7	6.7 <sup>d</sup>	3.4
Nucleus	31.9	0.34	0.3
Plastids	2.7 <sup>e</sup>	19.0	0.5 <sup>f</sup>
Mitochondria	4.2	1.0	0.5
Vacuoles	16.7	73 <sup>g</sup>	79 <sup>g</sup>
ER	1.9	–	–
Golgi cisternae	1.1	–	–

<sup>a</sup> Pea shoot apex.<sup>b</sup> Cf. Winter et al. (1993).<sup>c</sup> Cf. Winter et al. (1994).<sup>d</sup> Including small vesicles and organelles.<sup>e</sup> Proplastids.<sup>f</sup> Chloroplast stroma.<sup>g</sup> Epidermal cells: 99% (barley); 89% (spinach).

On the whole, compartmentation permits specialization and division of labour within a cell on a large scale, as well as optimal efficiency by concerted action, laying the basis for complex cellular morphogenesis, differentiation, and ultimately also for multicellularity (being characteristically confined to eukaryotes). In Table 1 the compartments of typical plant cells are enumerated (cf. Steer 1991; Leidreiter et al. 1995a,b). Some data on volume proportions of different compartments are given in Table 2. These proportions are subjected to a large degree of variation in cell differentiation.

After the discovery of intracellular compartments and their mass isolation from cell homogenates in the 1950s, research concentrated on the topology of metabolic reaction chains and cycles. The results of the corresponding studies have long since sedimented into textbooks. Today, compartmentology has experienced new impulses through the application of molecular biological methods and the renaissance of light microscopy, permitting analytical studies in living cells (cf. Harris and Oparka 1994; Clark 1997). Currently, research is focused on nucleus-encoded proteins, that are translated in the cytosol and specifically translocated into the different compartments of the cell, as well as on biogenesis and the phyletic evolution of compartments. Some results of studies in compartmentation have led to concepts which have revolutionized the picture of "the" cell. However, some older yet still valid conjectures are no longer remembered. It seems, therefore, appropriate to outline the present status of compartment research together with the background of older endeavors. Due to the enormous breadth of the topic, original papers can only be quoted in exceptional cases (e.g., if they are not covered by reviews, or if they deal with a problem with particular clarity).

## 2. Neither Membranes Nor Compartments Are Formed *de Novo*

According to textbook wisdom, biomembranes are smectic liquid crystals, built of amphipolar lipid molecules in the form of lipid bilayers that are traversed by integral membrane proteins and the surfaces of which are locally beset by peripheral proteins (cf. Op den Kamp 1994). Lipid bilayers can easily be produced artificially and they are commonly used in the form of black films or liposomes reconstituted with membrane receptors, translocators, or ion channels, to investigate questions of membrane transport or signal transduction. However, contrary to artificial lipid bilayers, biomembranes arise in living cells exclusively from already existing biomembranes by continuous incorporation of newly synthesized molecular constituents and not *de novo* (Luria 1973; Sitte 1977). Any statements on a *de novo* formation of, e.g., vacuoles or Golgi cisternae in reality mean that the respective compartments and their membranes come about by separation and differentiation from other membranes/compartments). Even where an instantaneous formation of "new" membranes appears most likely, as in the case of instantaneous formation of a "new" plasma membrane around naked drops of endoplasm of *Physarum*, a buildup of this membrane by rapid vesicle fusion has been demonstrated (Wohlfarth-Bottermann and Stockem 1970). Thus, biomembranes can easily enlarge in area by intussusceptional growth, they may differentiate by incorporation of new protein molecules, and they may become separated from each other by membrane flow; yet they cannot develop without preexisting biomembranes. In other words, biomembranes exhibit genetic continuity, and so do the compartments comprised by them, a fact that has important consequences for phylogenetic considerations and cell compartmentation (Sects. 11, 12).

Why can biomembranes not arise *de novo*? Even smallest membrane vesicles with not more than 50 nm diameter contain about 40,000 lipid molecules. However, irregular accumulation of amphipathic molecules might be deleterious for a cell. Furthermore, many enzymes essential in lipid synthesis are integral membrane proteins, as are specific receptors for membrane proteins. Finally, biomembranes are basically asymmetric whereas artificially formed lipid bilayers (even if reconstituted with integral membrane proteins) are symmetric in cross section.

## 3. Dynamics of Compartments

Bulk transport within cells by compartment separation, translocation, and fusion of compartments is accomplished by membrane flow (also termed vesicular traffic, or cytotic events). Transports of this kind can be either inwards-directed (endocytosis) or outwards-directed (exocytosis).

Corresponding processes have been known for a long time, as they can be observed by light microscopy in living cells and have been termed phagocytosis, pinocytosis, or potocytosis in the case of endocytotic events, and granulocrine secretion in the case of exocytosis. The study of membrane flow processes was dramatically intensified after the advent of the electron microscope (cf. Bennett 1956; Schnepf 1968, 1969 a,b; Palade 1975; Robinson and Kristen 1982), and again more recently by the application of molecular biological methods (Predroso de Lima et al. 1995; Cold Spring Harbor Symposia of Quantitative Biology LX 1995; Sweet 1977). It turned out that the underlying processes are basically similar in all eukaryotes and that many essential proteins are highly conserved (cf. Dobberstein 1994). Nevertheless, in plant cells the respective events exhibit some peculiarities that must be accounted for whenever extrapolations are attempted of results obtained with animal or yeast cells (cf. Gal and Raikhel 1993).

#### 4. Plasma Membrane and Endocytosis

The plasma membrane (PM) defines the living protoplast both morphologically and functionally. In plant cells the PM is fitted with the complexes of cellulose synthase and callose synthase (Delmer and Amor 1995; Robinson 1996a). It determines, by its characteristic complement of translocators and ion channels, the exchange of ions and compounds between the cell and its environment or, for that matter, between symplast and apoplast (Kristen 1989; Sussman 1994; Aidley and Stanfield 1996; Assmann and Haubrick 1996). Ion pumping ATPases which accomplish the outwards-directed transport of protons and calcium ions energize solute uptake by diverse solute transporters. (Contrary to animal cells,  $\text{Na}^+$ -ATPases play only a minor role in plant cells.) Prominent among the PM ATPases are the  $\text{H}^+$ -ATPases which maintain a relatively high pH in the cytosol (7.2–7.5) and energize many secondary transporters (Michelet and Boutry 1995). Contrary to tonoplast  $\text{H}^+$ -ATPases, the proton-pumping ATPases of the PM are insensitive to  $\text{NO}_2^-$ , yet are sensitive to vanadate. The plant cell PM  $\text{H}^+$ -ATPases, furthermore, exhibit a very narrow pH optimum around pH 7 (Hager et al. 1986). Plant  $\text{H}^+$ -ATPases are encoded by a multigene family. Recently, three isoforms of *Arabidopsis* PM ATPases (AHA1–3) were cloned and expressed in the yeast system (Regenberg et al. 1995). The  $\text{H}^+$ -ATPases of the PM of guard cells mediate the opening of the stomata in response to diverse stimuli (light, auxin, 1,2-diacylglycerol, fusicoccin; cf. Assmann 1993), by activation of inward-rectifying, voltage-gated  $\text{K}^+$  channels. Conversely, closing of the stomata is brought about by the efflux of potassium and anions after an increase of the cytosolic calcium concentration (MacRobbie 1992). It has been shown repeatedly that the proton AT-

Pases of the PM of guard cells and mesophyll cells are reversibly inhibited by submicromolecular concentrations of cytosolic  $\text{Ca}^{2+}$  (Bush 1995; Chasan 1995a; Kinoshita et al. 1995).

Transport systems of the PM that are energized by proton ATPases (cf. Maathuis and Sanders 1992; Tanner and Caspari 1996) include sugar transporters (Sauer and Tanner 1993),  $\text{K}^+$ -channels (Bentrup 1989; Sussman 1992; Jan and Jan 1994; Cao et al. 1995; Rubio et al. 1995; Hedrich and Dietrich 1996), a  $\text{K}^+/\text{H}^+$  cotransporter (Schachtman and Schroeder 1994), as well as transport systems for amino acids and peptides, for  $\text{NH}_4^+$ , and for sulfate. Hydraulic conductivity is conferred to the PM by water channels (Brown et al. 1995). These are formed by membrane proteins with 6 transmembrane domains and allow up to  $4 \times 4^9 \text{ H}_2\text{O}$  molecules/s to pass through. Channels of this kind (aquaporins) were first discovered in mammalian cells. Yet, they occur in all eukaryotes in several isoforms (Chrispeels and Agre 1994; Robinson et al. 1996a). In plant cells they have been termed TIPs or PIPs, respectively (*tonoplast* or *plasma membrane intrinsic proteins*, cf. Kammerloher et al. 1994; Kaldenhoff et al. 1995; Robinson et al. 1996b; for TIPs, see Sect. 6). Since  $\text{HgCl}_2$  and  $\text{ZnCl}_2$  impede water permeation, thiol groups seem to be involved (Tazawa et al. 1969). The water conductivity is inwards greater than outwards ("polar water permeability").

In quite a few cases, the area of PM is enlarged either by cell wall protuberances or so-called plasmatabules (Kristen 1989), indicating intensive exchange between apoplast and symplast. It is possible, though not proven, that these structures are analogous to the "caveolae" of certain animal cells, small invaginations of the PM that do not become detached from it and which are thought to facilitate exchange and signal transduction (Parton 1996).

The PM not only serves important transport processes but also is the site where endocytotic vesicular traffic commences (potocytosis, phagocytosis. On myzocytosis, which is restricted to certain parasitic dinoflagellates that feed on single cells, cf. Schnepf and Deichgräber 1984). Endocytosis is of general occurrence in animal and human cells and many protocists, whereby certain cell types are specialized for it (cf. M. S. Robinson et al. 1996). It has also been demonstrated for plant protoplasts, walled plant cells (Hübner et al. 1985), and yeast cells (cf. Robinson and Hillmer 1996a; Riezman 1993; Low and Chandra 1994).

Endocytosis starts with the adsorption of (macro-)molecules at the outer face of the PM by specific receptors (Braun and Walker 1996; Lamb 1996), followed by formation of coated pits (Robinson and Hillmer 1990b), and pinching-off of coated vesicles (CV; Robinson and Depta 1988), which in plant and yeast cells are normally delivered to the vacuole. The coat of the endocytotic vesicles (and also of vesicles in the membrane flow between Golgi dictyosomes and vacuoles) is built of

clathrin and accompanying proteins ("clathrin-coated vesicles", Pearse and Robinson 1990; M. S. Robinson 1994; Robinson 1996b). Coat formation affords the assistance of adaptins (Robinson 1992; Drucker et al. 1995; Traub 1997). After vesicle formation and before fusion of the vesicles with tonoplasts, the coat must be disassembled with the help of chaperone hsc70 ("uncoating ATPase") and the 100-kDa protein auxilin (Ungewickell et al. 1995). Certain steps in endocytosis are energized by GTPases. Small GTPases belong to the Rab family (Marsh and Cutler 1993; Novick and Garrett 1994). Dynamin is a large GTPase with more than 800 amino acid residues which serves several functions and also plays an important role in endocytosis (Trowbridge 1993; Kelly 1995; Vallee and Okamoto 1995).

An uninterrupted PM is absolutely indispensable for every cell. In tissues growing by cell multiplication the PM must be able to enlarge infinitely. The enlargement results primarily from the incorporation of exocytotic vesicles. Under steady-state conditions of nongrowing cells, exocytotic activities must be counterbalanced by endocytosis, which also seems to be important for maintaining the specific composition of the PM (Allan and Kallen 1994).

## 5. Plasmodesmata

Although cells in multicellular organisms are separate entities, there is a steady exchange of ions, molecules, and signals between adjacent cells (symplastic transport) which are often electrically coupled (Gunning and Robards 1976). In plant tissues, plasmodesmata (PD) are the main mediators of symplastic transport (Robards and Lucas 1990; Lucas and Wolf 1993; Lucas 1995; see also Ap Rees 1994; on the isolation and biochemical characterization of PD cf. Monzer and Kloth 1991; Tilney et al. 1991). "Primary" PD are formed during cell plate formation. If no further PD are generated, their density in growing walls decreases (Gunning 1978). Normally, however, additional "secondary" PD are formed in growing cell walls (cf. Schnepf and Sych 1983). PD are also found in walls between cells that were originally separated (Binding et al. 1987; Monzer 1991; van der Schoot et al. 1995), and even between cells from different species or genera (Kollmann and Glockmann 1985; Kollmann 1992; Steinberg and Kollmann 1994; Dörr and Kollmann 1995). In such cases the cell walls become enzymatically thinned and eventually perforated at corresponding sites of adjacent cells; after endoplasmic reticulum (ER) connections have been formed the cell wall is regenerated (Kollmann and Glockmann 1991).

The functioning of PD depends on their permeability for ions and molecules. Except in the case of sieve pores (which are excessively enlarged PD), symplastic transport takes place in the narrow cytoplasmic



sleeves between the PM that confines the single PD as a hollow cylinder, and a central proteinaceous desmotubulus (Tilney et al. 1991). The cytoplasmic sleeves are narrowed by sphincter structures at the neck regions on both ends of a PD which presumably diminish the porosity of the PD. A meshwork of linking proteins connects the desmotubule with the PM, further reducing PD permeability by forming irregular microchannels of about 2.5 nm diameter. Typically, particles of up to 1 kDa can pass through PD. However, this value varies greatly. By virtue of special gating factors, much larger particles (up to more than 20 kDa) may pass through PD. Better known examples are nucleic acids, proteins, dextrans, and virions (cf. Wolf et al. 1989; Lucas and Wolf 1993; Waigmann and Zambryski 1994, 1995; Zambryski 1995; Sanderfoot and Lazarowitz 1996). On the other hand, PD may also be entirely occluded (for an extreme case, cf. Schnepf and Sawidis 1991). Correspondingly, estimates of transport capacities by plasmodesmograms (diagrammatic representation of PD density in cell walls) are in most cases of only limited value (van Bel and Oparka 1995).

Animal tissues are devoid of PD, but adjacent cells are often connected by gap junctions (Robards et al. 1990; Wolburg and Rohlmann 1995; Kumar and Gilula 1996): Hexagonal complexes (connexons) of integral membrane proteins (connexins) form hydrophilic channels which can transport particles up to 1 kDa. In gap junctions, numerous connexons are arranged in hexagonal arrays. PD and gap junctions (or connexons) are functionally analogous despite their great structural differences. Nevertheless, in extracts of plant tissues, polypeptides could be demonstrated which react with specific anticonnexin antibodies (Meiners et al. 1991; Hunte et al. 1992; Janßen et al. 1994). The cross-reacting proteins are probably localized in the neck region of PD (Yahalom et al. 1991; Schulz et al. 1992). This (putative) protein homology between connexins and PD proteins is surprising since the phyletic separation of animals and plants took place, according to present knowledge, at the niveau of unicellular organisms.

## 6. Vacuoles and Tonoplasts

Large vacuoles, comprising up to 99% of the protoplast volume, are characteristic for plant and fungal cells. (Contractile or pulsating vacuoles will not be considered here.) The functions of vacuoles vary widely (Marmé et al. 1982; Wink 1993; Robinson and Hinz 1997) and a single cell often contains vacuoles with different functions. These functions are reflected by the transport proteins of the vacuolar functions are producing cell turgor and performing hydrolysis of macromolecules ("vegetative" and "lytic" vacuoles, respectively), or storage of different material, including seed proteins.

- Turgor is generated and maintained by active transport processes at the tonoplast (Barkla and Pantoja 1996; on ion pumping ATPases see next paragraph) that have been studied particularly in the guard cells of stomata (cf. Raschke et al. 1988; Schroeder and Hedrich 1989; Assmann 1993; Ward and Schroeder 1994; Allen and Sanders 1994, 1995; Chasan 1995a; Schulz-Lessdorf and Hedrich 1995). The presence of water channels (aquaporins, TIPs, cf. Sect. 4) has amply been demonstrated for tonoplasts (Chrispeels and Agre 1994; Chrispeels and Maurel 1994).
- Vacuoles are filled with an acidic cell sap and certain acid hydrolases. Soon after the establishment of the lysosome concept, vacuoles therefore were envisaged as the lytic compartments of plant cells (Matile 1975, 1978; Leigh 1979; Chiang and Schekman 1991). Proton-pumping ATPases of the tonoplast, belonging to the V-type of membrane ATPases (Harvey and Nelson 1992), together with tonoplast pyrophosphatases that make use of inorganic pyrophosphate as energy source, are responsible for the low pH (Hoffmann and Bentrup 1989; Maathuis and Sanders 1992; Rea et al. 1992). Both proton pumping complexes can be identified in freeze-etch preparations of tonoplasts (Mariaux et al. 1994). The V-ATPases are structurally similar to mitochondrial F-ATPases which function in vivo as ATP synthases (cf. Klink and Lüttge 1991; Taiz and Taiz 1991; Getz and Klein 1995). The amino acid sequences of the 60- and 70-kDa subunits of V-ATPases exhibit a certain degree of homology to  $\alpha$  and  $\beta$  subunits of mitochondria/eubacterial  $F_0F_1$ -ATPases, yet homology to corresponding subunits of the archaeon *Sulfolobus* is considerably higher (Gogarten et al. 1989; Nelson and Taiz 1989; Nelson 1992), an interesting fact with regard to the phyletic evolution of organelles (see Sect. 11). Tonoplast ATPases amount to one quarter to one third of the total tonoplast proteins (Klink et al. 1990; Ratajczak et al. 1995). They are surpassed only by the tonoplast aquaporins (TIPs, see Sect. 4; Chrispeels and Agre 1994; Chrispeels and Maurel 1994).  $\gamma$ -TIPs prevail in the tonoplast of vegetative to protein-storing vacuoles (Johnson et al. 1989). The ingestion of cellular material for destruction in yeast vacuoles seems to come about partly by bulk transport, similar to autophagic events (Takeshige et al. 1992), or, alternatively, by direct import of proteins (Chiang and Schekman 1991; Klionsky et al. 1992) mediated by ABC transporters (part of their subunits contain an ATP-binding cassette, ABC; cf. Higgings 1992; Cleves and Kelly 1996).
- Storage material, but also (partly poisonous) end products of secondary metabolism, as well as certain ions are sequestered in vacuoles (Willenbrink 1987; Kreis and Hölz 1991). Among the inorganic ions concentrated in vacuoles,  $Ca^{2+}$  (Clarkson and Lüttge 1989; Kinzel

1989) and  $\text{Na}^+$  prevail, the latter particularly under salt stress. As in animal cells (cf. Lytton and Nigam 1992), the cytosolic concentration of calcium is kept very low (that is, in the micromolar range) to avoid disastrous precipitations of cytoplasmic calcium phosphate. This very fact renders possible the use of  $\text{Ca}^{2+}$  as a second messenger (Bush 1995). In the course of signal transduction, release of calcium ions from vacuoles through voltage, or inositol 1,4,5-trisphosphate-gated calcium channels, has been demonstrated (e.g. Alexandre et al. 1990; Pantoja et al. 1992; Allen et al. 1995). Besides inorganic ions, metabolites such as malate, oxalate, and sucrose, as well as hydrophilic pigment molecules are often accumulated in vacuoles. The transport of these compounds across the tonoplast is energized by membrane potentials or the low internal pH of vacuoles and is accomplished by specific translocators (Maathuis and Sanders 1992; Martinoia 1992; on patch-clamp studies with tonoplasts, cf. Hedrich et al. 1988; on malate transport in crassulacean acid metabolism (CAM) plants: Smith and Bryce 1992; Martinoia and Rentsch 1994; Kluge and Galla 1996). Heterocyclic xenobiotics are sequestered in vacuoles in the form of complexes with glutathion (Martinoia et al. 1993; Marrs 1996). In seeds and fruits the vacuoles are often storage compartments for proteins (see below).

Where do vacuoles and their membranes come from? As vacuoles are diverse and heterogeneous (cf. Paris et al. 1996), no simple answer can be expected to this question. Most often, vacuoles clearly stem from pre-existing ones. For example, large vegetative vacuoles arise through fusion of smaller vacuoles (which are present in every plant cell) during postembryonal cell growth. Conversely, large vacuoles can be dispersed into smaller vacuoles under certain conditions. However, can vacuoles also be formed *de novo* from other components of the endomembrane system? Most probably, yes. There are strong indications that vegetative vacuoles can come about by autophagic processes (Khera and Tilney-Bassett 1976). In the case of protein storing vacuoles of seed cells, *de novo*-formation from a complex tubular-cisternal membrane system has been reliably documented (Hoh et al. 1995). This type of vacuole may also arise directly from the ER without participation of the Golgi apparatus (cf. Kristen 1989; Rothman et al. 1989; Höfte and Chrispeels 1992; Müntz 1992; Gal and Raikhel 1993; Galili et al. 1993; Li et al. 1993; Geli et al. 1994; Hoh et al. 1995; Robinson et al. 1995). However, in other instances vacuolar components are delivered by the Golgi apparatus (Stack et al. 1995; Okita and Rogers 1996). Two diverse populations of vesicles that originate at the *trans*-Golgi network (TGN, see Sect. 8.c) seem to be engaged in supplying vacuolar proteins: clathrin-coated vesicles deliver hydrolases to lytic vacuoles, whereas "dense vesicles", devoid of a clathrin coat, transport proteins to storage vacuoles (Hohl et al. 1996; Robinson 1996b). At least for tonoplast proteins, an even more

complicated transport system involving the PM (secretion, followed by endocytosis, as described repeatedly for animal tissue cells) cannot be ruled out for plant cells, too (Robinson et al. 1996a; on a corresponding condition in yeasts cf. Armstrong 1991; Vida et al. 1993; Chapman 1994; Horazdovsky et al. 1995).

## 7. Microbodies and Peroxisomes

Microbodies can be specialized for quite different metabolic activities. They contain up to 40 different peroxisome-specific proteins (van den Bosch et al. 1992). Many of them are enzymes, mainly  $H_2O_2$ -generating oxidases as well as catalase. Microbodies of this kind are known as peroxisomes (cf. Subramani 1993; Leiper et al. 1995; Masters and Crane 1995; Olsen and Harada 1995).

In mesophyll cells, "*leaf-type*" peroxisomes serve as organelles for photorespiration: In C3-plants they carry out part of the recycling reactions of phosphoglycolate. Toxic intermediates ( $H_2O_2$ , glyoxylate) are immediately metabolized to water,  $O_2$ , and glycine. Surprisingly, the membrane of leaf peroxisomes is permeable for all of these compounds, as it contains porin-like channels (Reumann et al. 1995). Metabolic compartmentation and metabolic channeling is achieved by formation of compact multienzyme complexes (Heupel et al. 1991; Heupel and Heldt 1993). The high permeability of the membranes of leaf peroxisomes meets a functional requirement as the successive reactions of photorespiration are distributed among chloroplasts, peroxisomes, and mitochondria, which accordingly exhibit close topological relationships in mesophyll cells.

During germination of oil seeds the transient appearance of *glyoxysomes* is observed which accomplish the mobilization of storage lipids as well as gluconeogenesis. Contrary to the membranes of leaf and other peroxisomes, the membranes of glyoxysomes are not porous (Donaldson et al. 1981), yet they are fitted with specific translocators. In the methylo-trophic yeast, *Candida boidinii*, an integral membrane protein (PMP47) has been shown to be a major component of the peroxisomal membrane. Its amino acid sequence exhibits homology to mitochondrial and plasmal-tidal solute carrier proteins (Jank et al. 1993).

*Root nodule peroxisomes* perform the conversion of urate to allantoin in noninfected cells of the root. On the extremely variable peroxisomes of mammals, and human diseases caused by peroxisomal defects, cf. Latruffe and Bugaut 1993. The metabolism of glucose and glycerol normally takes place in the cytoplasm; in the Trypanosomatidae, however, the corresponding reactions are concentrated in special microbodies, the *glycosomes* (cf. Opperdoes 1988).

Concerning the biogenesis of peroxisomes, genetic studies with cells of mammals and yeasts (where formation and multiplication of peroxisomes can be induced by special nutritional conditions) have been particularly successful (Erdmann et al. 1989, 1991; cf. Leiper et al. 1995). The original assumption according to which peroxisomes were derived from the ER could be disproved (Lazarow and Fujiki 1985; Lazarow 1993). Rather, peroxisomes arise from the enlargement and division of preexisting peroxisomes (Borst 1989). Peroxisomal matrix proteins are synthesized on free cytoplasmic polysomes and are only posttranslationally translocated into peroxisomes. The translocation process affords ATP. The various peroxisomal targeting signals (PTS) of matrix proteins differ greatly from the ones for protein import into mitochondria and chloroplasts. Furthermore, there is no processing during protein translocation into peroxisomes. The carboxyterminal tripeptide SKL functions as the major PTS (PTS1; Banjoko and Trelease 1995). PTS2 is a conserved aminoterminal nonapeptide. Less frequently, internal sequence stretches could also be determined as PTS (Subramani 1993; 1996; Olsen and Harada 1995). The PTS receptors are currently under study (cf. Purdue and Lazarow 1994; Rachubinski and Subramani 1995). Little is known about the translocation complexes. Molecular chaperones or unfoldases do not seem to be involved, since folded proteins as well as protein oligomers and even 9 nm gold particles coated with peroxisomal proteins are translocated into peroxisomes (McNew and Goodman 1996). Surprisingly, even proteins without a PTS are transferred into peroxisomes in the form of complexes with PTS-containing proteins. The enzyme complement of a given peroxisome type apparently does not come about by import selection at the peroxisomal membrane. Ectopically expressed proteins in transgenic plants are translocated into peroxisomes of tissue cells in which they otherwise would not occur (Olsen et al. 1993; Onyeocha et al. 1993). A gradual transformation of glyoxysomes into leaf peroxisomes (and reverse in senescing cotyledons: Nishimura et al. 1993) has been repeatedly described.

The membrane proteins of peroxisomes are also translated on free polysomes in the cytoplasm. However, their targeting signals differ from the ones of matrix proteins. Membrane enlargement and production of matrix material appear to be regulated separately. In mutant yeasts, but also in certain diseased human cells, an accumulation of aberrant peroxisomal "ghosts" has been observed. In these cases, the peroxisomal membranes are apparently unable to import newly synthesized matrix proteins (Lazarow 1993; Motley et al. 1994).

Peroxisomes provide an impressive example of DNA-free compartments which nevertheless exhibit genetic continuity as they are strictly *sui generis*.

## 8. ER, Golgi Apparatus (GA), and the Exocytotic Membrane Flow

### a) ER and Cotranslational Protein Transport

The exocytotic membrane flow (cf. Steer 1991) commences at the ER, which is a prominent compartment in most eucytes and is marked by a high surface-to-volume ratio. Essential steps of membrane lipid synthesis are located at the ER (Somerville and Browse 1996). Furthermore, co- or posttranslational incorporation of integral membrane proteins as well as the synthesis of lysosomal (vacuolar) and secretion proteins take place at the rough endoplasmic reticulum (rER). In the past 20 years, the signal hypothesis of Blobel and Dobberstein (1975) has been amply confirmed; the molecular structure of the signal recognition particles (SRP) can be found in every textbook. Many of the varied targeting signals have been discovered. Presently, the interest is focused on details of targeting, on the receptors for SRP and ribosomes at the rER, and on the very nature of the pores for the vectorial discharge of nascent polypeptides into the lumen of the ER (Kreibich and Sabatini 1992; Rapoport 1992; Vitale et al. 1993; Johnson 1993, 1997; Walter and Johnson 1994; Wolin 1994). Fortunately, the proteins involved in these processes are highly conserved so that results obtained with particularly suitable systems may cautiously be generalized (Dobberstein 1994; Jungnickel et al. 1994; Wolin 1994).

A heterodimeric cytosolic protein complex in the cytosol (termed NAC, for nascent polypeptide-associated complex; Lauring et al. 1995) helps to avoid mistargeting of ribosomes with nascent polypeptides devoid of a targeting signal, which otherwise should take place due to the high affinity of ribosome receptors at the ER membranes. The NAC binds to nascent peptides whereby signal sequences are left free. The structure of pores ("translocons") in the ER membrane through which proteins are totally or partly translocated could be resolved by photochemical or chemical cross-linking of nascent secretory proteins to integral membrane proteins (cf. Gilmore 1993; Görlich 1994; Jungnickel et al. 1994; Andrews and Johnson 1996; Martoglio and Dobberstein 1996; Rapoport et al. 1996). As postulated by Blobel and Dobberstein already in 1975, there is a hydrophilic channel which is, however, sealed off towards the ER lumen and also towards the large subunit of the affixed ribosome so that the permeability barrier is maintained. The channel is formed by a membrane protein homologous to the  $\alpha$ -subunit of Sec61p (yeast) or SecY (*E. coli*), respectively. This protein is, at the same time, the long-looked-for ribosome receptor. Most recently, the translocon could be demonstrated in the EM: 3–4 Sec61p trimers form a cylindrical complex with a central channel of about 2 nm diameter (Hanein et al. 1996; Schekman 1996). Connected with this complex are the SRP receptor, the signal peptidase, the TRAM protein (translocating chain-

associating membrane protein; Görlich et al. 1992), an oligosaccharyl transferase, and presumably also the two ribophorins which for a while were assumed to be the ribosome receptor. Translocation is energy-dependent, and GTPases are involved in this process (Gilmore 1993; Miller et al. 1993; Dobberstein 1994).

In the lumen of the rER there is a high, that is millimolar,  $\text{Ca}^{2+}$  concentration (buffered by calcium-binding proteins such as calreticulin; cf. Denecke et al. 1995) and redox conditions which favour spontaneous formation of disulfide bonds. The main redox buffer in the ER appears to be glutathione. Whereas the ratio of oxidized to reduced glutathione in the cytosol is approx. 1:100, it is 1:10 in the ER lumen (Hwang et al. 1992). Correct folding of secretion proteins is achieved by several molecular chaperones and disulfide isomerase (Helenius et al. 1992). In the ER lumen, numerous soluble proteins are present (collectively termed reticuloplasmins) which either remain in the ER or are (more likely) retrieved from the *cis*-Golgi. Reticuloplasmins possess a retention signal in the form of a carboxyterminal tetrapeptide (in yeasts and plants most often HDEL (histidine-aspartic acid-glutamic acid-leucine), in animals mainly KDEL; cf. Pelham 1990, 1995; Vitale et al. 1993; Nilsson and Warren 1994). In *Arabidopsis*, homologues to the animal KDEL (lysine-aspartic acid-glutamic acid-leucine) receptor (an integral membrane protein with seven transmembrane domains) could be demonstrated (Bar-Peled et al. 1995).

## b) Vesicle Traffic

From the ER to the *cis* side of Golgi dictyosomes as well as between the *trans* side of dictyosomes and the PM (and probably also within the Golgi stacks), secretory products are transported in bulk form by vesicles. Investigations on particularly promising systems such as neuronal synapses, virus-infected tissue culture cells and yeasts resulted in detailed conjectures on the budding of transport vesicles from donor membranes, their fusion with acceptor membranes, and the proteins involved in these processes (Johannes 1994; Rothman 1994; Kreis et al. 1995; Whiteheart and Kubalek 1995; Bednarek et al. 1996; Fabry 1996; Schekman and Orci 1996; Robinson 1997). Diverse inhibitors proved to be invaluable tools in dissecting the chain of successive events, among them the cysteine-alkylating agent N-ethylmaleimide (NEM), nonhydrolyzable analogs of GTP and ATP, and brefeldin A (BFA; cf. Orci et al. 1991; Robinson 1993; Satiat-Jeunemaitre and Hawes 1994). Currently, the prevailing picture is as follows.

The formation of transport vesicles at the ER is initiated by attachment of small G-proteins that have previously exchanged GDP against GTP by help of further factors. An early discovered example of the G-

proteins involved is Arf (ADP ribosylation factor; cf. Donaldson and Klausner 1994; in yeast this is identical to Sar1p). GDP/GTP exchange is inhibited by BFA (Ferro-Novick and Novick 1993). After attachment of Arf an increased content of phosphatidic acid and phosphatidylinositol metabolites can be observed in the donor membrane (De Camilli et al. 1996, Seaman 1996). Furthermore, cytosolic complexes of coat proteins (COPs), so-called coatomers, become attached to the membrane. Two different species, COPI and COPII, have been identified (and can be distinguished in the EM; Seaman and Robinson 1994). These processes lead eventually to vesicle budding and the separation of the vesicle from the donor membrane.

After translocation to acceptor membranes (presumably assisted by cytoskeletal elements) the vesicles fuse with the latter. As a prerequisite for fusion, the vesicle coat must be shed. This is effected by hydrolysis of (Arf-)-GTP, a process inhibited by GTP $\gamma$ S. For actual membrane fusion, a cytosolic factor is necessary which can be blocked by NEM. This factor has been termed NSF (NEM-sensitive fusion protein; in yeast it is identical to Sec18p). NSF exhibits ATPase activity, and ATP hydrolysis is in fact necessary for the fusion of vesicle and acceptor membranes. As a soluble protein, NSF does not bind directly to membranes; rather, it must be fixed by (likewise soluble) NSF attachment proteins (SNAPs). The NSF-SNAP complex then becomes linked to the vesicle membrane and in this form is recognized by integral SNAP receptors (SNAREs) at the target membrane by help of Rab GTPases (Pfeffer 1994; Bennett 1995; Aridor and Balch 1996a). Such ligand/receptor interactions among v-SNAREs and t-SNAREs ensure specificity in vesicular traffic (the "SNARE hypothesis" is connected mainly with the names of James E. Rothman, Lelio Orci, and Randy Schekman; cf. Söhlner et al. 1993). The SNARE hypothesis has been greeted as a long-desired unifying concept and it has been propagated with impressively coloured schemes, although some open questions still remain (as, for example, the selection of vesicle content: Aridor and Balch 1996b) and also some seeming or real inconsistencies (cf. Balch and Farquhar 1995; Griffith et al. 1995; Singer 1995). The question of whether the whole concept may be generalized is still open. Nevertheless, the enormous heuristic value of the SNARE hypothesis is beyond doubt. On its basis, interesting concepts could be developed on the structural dynamics of compartments (transformation of cisternae to tubular networks and finally to vesicles: Rothman and Warren 1994; Denesvre and Malhotra 1996).

### c) GA, Dictyosomes, and Exocytosis

The GA of plant cells (Staehelin and Moore 1995) is characterized by numerous dictyosomes that are scattered over the entire cytoplasm



("dispersed GA"). Contrary to animal cells, where the Golgi cisternae vesiculate during mitosis (Warren 1989; Warren et al. 1995), the Golgi stacks in plant cells remain intact in the course of cytokinesis and are actively involved in cell plate formation (Dupree 1996; Staehelin and Hepler 1996). The fact that cytokinesis can be achieved in eucytes either by means of contractile actomyosin rings (as in zoocytes) or by intracellular formation of a new separating wall (as in phytocytes) reflects a fundamental difference between animal and plant cells. Likewise, protein secretion, playing a prominent role in certain animal cell types, is quite less pronounced in plant cells where Golgi dictyosomes are engaged primarily in the synthesis of cell wall polysaccharides. O-glycosylation of typical cell wall proteins such as hydroxyproline-rich glycoproteins (HRGPs) and arabinogalactan proteins (AGPs), as well as the synthesis of pectic substances and hemicelluloses, are characteristic functions of the plant Golgi compartment. Nevertheless, and corresponding to the situation in animal cells, N-glycosylation of membrane proteins and exoenzymes commences in the ER and is only completed in dictyosomes. Contrary to the situation in animal cells, however, terminal sialic acid residues are lacking in the heterosaccharide chains of N-glycosylated plant glycoproteins.

In the vast majority of plant and fungal cells, the Golgi stacks exhibit a distinctive morphological and functional *cis* → *medium* → *trans* polarity. As in animal cells, the outermost cisterna on the *trans* side is often organized as a tubular or reticulate "trans-Golgi network" (TGN). At the TGN, Golgi vesicles are formed for carrying cell wall material to the PM, and clathrin-coated vesicles bud off from the TGN for bulk transport towards vacuoles. The TGN is the principal sorting compartment in the exocytotic membrane flow.

A still open question concerns the *cis* → *trans* transport of macromolecular cargo within the Golgi stack. In the "cisternal progression model" it is postulated that in *cis*, new Golgi cisternae come about by fusion of ER-derived vesicles, whereas *trans* cisternae are used up in the production of Golgi vesicles (cf. the older terms "forming face" and "secretion face"). According to this model, a single Golgi cisterna moves down the Golgi stack step-by-step from *cis* to *trans*. The "vesicle shuttle model", on the other hand, envisages stationary Golgi cisternae and a transport of secretory products by vesicular traffic on the periphery of the dictyosome. In the yeast system, the involvement of v-SNAREs and t-SNAREs for traffic through the Golgi complex has been demonstrated (Banfield et al. 1995). At present, however, neither model is entirely satisfying since contradictory data exist for both of them (Becker and Melkonian 1995).

Another open question refers to retention or retrieval of certain Golgi components in the steady flow of compartments and their respective contents. This problem is currently the subject of intensive investigation

(cf. Luzio and Banting 1993; Wilsbach and Payne 1993; Pelham 1995; Cole et al. 1996).

Golgi vesicles travelling by active participation of the cytoskeleton (Geitmann et al. 1996) towards the PM and fusion with it deliver their contents into the extracellular space ("granulocrine secretion": Schnepf 1996a). Integral proteins of the vesicle membrane become proteins of the PM (at least transitorily, as they can be internalized again by endocytotic processes: Hager et al. 1991). The rate of exocytosis correlates with the cytoplasmic calcium concentration (Thiel et al. 1994; Derksen 1996). In plants and fungi, wall extension and cell growth depend on the rate of exocytosis which is correspondingly regulated by auxin (Schindler et al. 1994).

## 9. Nuclear Pore Complexes

The nuclear envelope (NE), a specialized cisterna of the ER (Dessev 1992; Dingwall and Laskey 1992; Goldberg and Allen 1995; Marshall and Wilson 1997), separates the compartments of transcription and translation. As a consequence, independent regulation of these fundamental processes is possible in eucytes. Also, some other familiar facts depend on the existence of an NE, among them the exon/intron structure of eukaryotic genes, intranuclear (and alternative) splicing, as well as the modification and storage of transcripts and the retention of transcription factors in the cytoplasm until the corresponding hormonal signals have been received. Recently, interest has been focused on nuclear pore complexes (NPCs) through which proteins and ribonucleoprotein particles (RNPs) are actively transported across the NE (Forbes 1992; Newmeyer 1993; Panté and Aebi 1994; Hicks and Raikhel 1995; Melchior and Gerace 1995). NPCs possess a total mass of 125 MDa (30 times the mass of a cytoribosome) and exhibit a highly symmetrical structure consisting of ca. 100 diverse proteins ("nucleoporins" that are often modified by glycosylation: Heese-Peck et al. 1995). Detailed models of the NPC have been designed in recent years on the basis of EM investigations of vertebrate nuclear envelopes and mass isolation of yeast NPCs (Rout and Blobel 1993; Panté and Aebi 1994; Rout and Wentz 1994).

How permeable are NEs, how specific is transport through NPCs, and how is it regulated and energized? Questions of this kind can be answered today, at least for the better studied nuclei of mammals, amphibians, and yeasts. Small particles of less than 40 kDa can diffuse freely across NPCs if only there is enough calcium in the perinuclear cisterna (Stehno-Bittel et al. 1995). Active translocation of larger proteins or RNPs affords ATP hydrolysis. In the course of such translocations, a central channel of up to 30 nm diameter is opened in NPCs. However, this does not seem to impair the barrier function of the NE, since a low

calcium concentration is steadily maintained within the nucleus as compared with the cytoplasm (Al-Mohanna et al. 1994), and  $K^+$  selective channels have been demonstrated in the nuclear envelope (Mazzanti et al. 1990). The accumulation of "karyophilic" proteins in the nuclear compartment has been alternatively explained, though, by facilitated diffusion across the NE and intranuclear complexation (Paine 1993). Thanks to investigations with fluorescence-labeled nuclear proteins in digitonin-permeabilized cells, the following picture of nucleo-cytoplasmic exchange can be drawn (cf. Fabre and Hurt 1994; Hurt 1996; Palacios and Izaurralde 1997):

- Import of karyophilic proteins from the cytoplasm is separated into two successive steps: (1) Recognition and attachment of proteins at the cytoplasmic side of the NPC (ATP-independent); and (2) ATP- or GTP-dependent transport through the NPC.
- Karyophilic proteins possess an NLS (nuclear location sequence) that is not removed during or after transport. It consists of a concentration of basic amino acid residues, often split into two separate partial sequences (Dingwall and Laskey 1991).
- NLSs are specifically recognized by cytoplasmic NLS receptors, called karyopherins. To date two types of karyopherins have been characterized: Importins (karyopherins  $\alpha$  and  $\beta$ : Adam 1995; Sweet and Gerace 1995; Görlich et al. 1995 a,b), and transportin (Aitchison et al. 1996; Pollard et al. 1996; cf. Dingwall 1996).
- Karyophile-karyopherin complexes first bind to the distal ends of filaments which extend from the NPCs into the cytoplasm. These filaments then bend to the center of the NPC, thereby transferring the complexes onto the cytoplasmic entrance site of the NPC. The corresponding processes have been made visible in the EM with gold-labeled nuclear proteins at NEs of *Xenopus* oocytes (Panté and Aebi 1996).
- After docking, the karyophile-karyopherin complexes are translocated into the nuclear compartment where they dissociate (Melchior et al. 1993; Moore and Blobel 1993, 1994). The translocation process affords hydrolysis of GTP, that is effected by a small G-protein (Ran/TC4) belonging to the Ras superfamily. Ran/TC4 binds, together with a small essential protein (p10: Nehrbass and Blobel 1996) to a large nucleoporin with 3324 amino acid residues (Moore 1995; Yokoyama et al. 1995). The translocated nuclear proteins remain in the nucleus whereas the other components shuttle back to the cytoplasm (Koepp and Silver 1996).
- Recently, specific nuclear export signals (NESs) could be demonstrated for the transport of proteins or RNPs out of the nucleus (Fischer et al. 1996; Moore 1996; Murphy and Wentz 1996).

Taken together, these findings show that in nucleocytoplasmic exchange specific and active transport processes prevail. The situation is reminiscent of the one encountered in plasmodesmata: In both cases a real barrier is set up and maintained between adjacent compartments despite the existence and activity of exchange sites of electron microscopic or even light microscopic dimensions. That means that specific unidirectional transport of macromolecules between separate compartments is possible, whereas, at the same time, concentration differences of quite smaller components can be reliably sustained.

## 10. Mitochondria and Plastids as Complex Compartments. Chemi-osmotic Energy Coupling

### a) Chemi-osmotic Theory

The chemi-osmotic theory by Peter Mitchell (Mitchell 1961; Mitchell and Moyle 1967; Skulachev 1984) is one of the most prominent concepts in compartmentology. It postulates intact compartment membranes, impermeable for protons, and containing integral reversible ATPases as a prerequisite for oxidative and photophosphorylation. In vivo, the ATPases function exclusively as ATP synthases that are energized by proton gradients and membrane potentials, which in turn are brought about by electron transport in the same membranes. The basic tenets of the theory can be found in every textbook, and many details have been clarified in recent years (cf. Ferguson 1995). The essential molecular components of the proton pumps and ATP synthases have been elucidated down to almost atomic dimensions (on cytochrome c oxidase cf. Calhoun et al. 1994; Iwata et al. 1995; Gennis and Ferguson-Miller 1996; on  $F_1F_0$ -ATPase = ATP synthase cf. Nelson 1992; Abrahams et al. 1994; Capaldi et al. 1994; Pedersen 1994).

### b) Protein Transport Machinery of Mitochondrial Membranes

The vast majority of mitochondrial membrane and matrix proteins are nuclear-encoded and are translocated posttranslationally into the organelles. The outer and inner mitochondrial membranes (*mom* and *mim*, respectively), that can be isolated and studied separately in the form of vesicles, are fitted with translocase complexes for protein (translocase of outer and inner mitochondrial membranes [TOM and TIM] respectively) that cooperate in vivo, although they are also functional upon separation (Kubrich et al. 1995; Ryan and Jensen 1995; Lill and Neupert 1996; Lill et al. 1996; Stuart and Neupert 1996; on plant mitochondria cf. Moore et al. 1994). More than 20 different proteins

have been identified as components of these complexes. Most of them have already been characterized functionally, particularly in *Saccharomyces* and *Neurospora*. The nomenclatoric chaos (each group had its own hieroglyphs) has been eliminated recently by compulsory rules (Pfanner et al. 1996). Accordingly, translocase components of the *mom* are abbreviated as Tom (for translocase of outer mitochondrial membrane) plus molecular mass in kilodaltons, and *mim* translocases correspondingly as Tim. If desired, the organism species may be indicated by a small letter prefix (e.g., yTom20 or pTom20 for the respective homologous components of TOMs from yeast and potato).

Protein import into mitochondria ensues as follows. The diverse targeting signals of freshly synthesized preproteins are recognized in the cytoplasm by chaperones of the Hsp70 family or by a dimeric mitochondrial stimulation factor (MSF) and are bound in suitable conformation. Two receptor complexes are positioned at the cytoplasmic surface of the *mom*, both of which are heterodimers (Tom37 + Tom70, and Tom20 + Tom22, respectively). Preproteins complexed with MSF (amongst them the proteins of the ADP/ATP-carrier family; Hachiya et al. 1994) are first bound by Tom37 + Tom70, then they are transferred to the Tom20 + Tom22 complex after shedding the MSF by ATP hydrolysis. The majority of mitochondrial preproteins bind directly to this complex, thus avoiding the Tom37 + Tom70 complex. These preproteins bear, as eliminative targeting signals, several basic amino acids at the N-terminus. Interestingly, the precursors of Tom20, Tom70 and Tom6 become incorporated into the *mom* without functional receptors, presumably by direct interaction with Tom38. Tom22 exhibits domains with an accumulation of negatively charged amino acid residues on both the extra- and intraplasmatic side of the *mom* which offer putative binding sites for targeting sequences. Accordingly, it is assumed that the N-termini of preproteins are translocated *cis*  $\rightarrow$  *trans* without ATP hydrolysis (Mayer et al. 1995). However, complete translocation of mitochondrial preproteins into the intermembrane space only with TOMs is not possible. Now the targeting sequence is received by a TIM. Transport across the *mim* affords ATP and a membrane potential  $\Delta\psi$ , as well as assistance by the mitochondrial mHsp70p. Supposedly, Tim17 and Tim23 form a passive channel and the transport is energized by the matrix ATPase mHsp70, Tim44, and  $\Delta\psi$ . Tim22 is indispensable for the incorporation of the mitochondrial ADP/ATP carrier into the *mim* (Sirrenberg et al. 1996).

The respective permeabilities of *mom* and *mim* are different. The *mom* can be passed by molecules up to ca. 0.8 kDa. This membrane contains porins which originally became known as voltage-dependent anion channels (VDAC) since they are, in their "open" state, slightly anion-selective. Porins are integral homotrimers, each subunit of which forms a diffusion pore with an antiparallel  $\beta$ -barrel structure (cf. Schulz

1996). Porins are highly variable regarding their amino acid sequences; no homology with bacterial porins could be demonstrated. As compared with the *mom*, *mim* is quite less permeable. However, it is fitted with diverse translocator proteins which mediate specific metabolite transport and the ATP/ADP exchange. Interestingly, certain nuclear-encoded tRNAs are imported into mitochondria by unknown means (Dietrich et al. 1992).

### c) Protein Translocation into Chloroplasts

Compared with the situation in mitochondria, the compartmentation of chloroplasts is complicated by the existence of a nonplasmatic thylakoid lumen, in addition to the nonplasmatic envelope intermembrane space, and the plasmatic stroma. Genetic studies (as in mitochondria) have not yet been possible. (Yeasts unfortunately lack plastids, thus impeding progress in botany.) Surprisingly, there are only minor similarities among the protein import machineries of mitochondria and plastids. Protein import into isolated chloroplasts was first demonstrated nearly 20 years ago, and about 10 years ago contact sites between the outer and inner envelope membranes (*com*, *cim*) were discussed for the first time as transport sites. However, proteins of the import apparatus of chloroplasts (IAPs) could only be identified recently by chemical cross-linking and after the isolation of translocating complexes (cg. Chrispeels et al. 1995; Gray and Row 1995; Schnell 1995; Kessler and Blobel 1996; Lübeck et al. 1996; Ma et al. 1996; earlier investigations were surveyed by Keegstra et al. 1995). Currently, the following picture of protein translocation into the diverse subcompartments of chloroplasts can be drawn (if only with some uncertainties).

Nuclear-encoded stroma proteins possess expanded N-terminal targeting sequences of between 35 and more than 100 amino acid residues. These are quite variable although hydroxylated and aromatic residues prevail (Chasan 1995b). The preproteins pass the envelope unfolded, with the N-terminus first. Transit sequences are eventually split off by a 150-kDa stromal transit peptidase. ATP and GTP, but no membrane potential, are necessary for transit (Theg and Scott 1993). The import receptor at the *com* is an integral 86-kDa protein. The preprotein is then transferred under ATP hydrolysis to the integral 75-kDa protein (IAP75) which, much as IAP86, but contrary to most of the other *com* proteins, contains an N-terminal presequence (Tranel et al. 1995). Thanks to membrane-spanning  $\beta$ -strands, this protein probably forms a channel for protein translocation. An integral IAP34, exhibiting GTPase activity, is tightly associated with IAP75 which, much as IAP86, possesses receptor capability. It is devoid of a presequence and is anchored with its C-terminus in the membrane, whereas a large part of it projects into the

cytoplasm (Seedorf et al. 1995). The *com* translocating complex also comprises an HSP70-related protein at the inner side of the *com* which receives the incoming polypeptide chain and is responsible for unidirectionality of the transport.

In the *cim* translocation complex, a 97- to 100-kDa protein plays a prominent role together with a 36-kDa and a 44-kDa protein. The large protein presumably recognized the targeting sequence and forms the membrane channel. The stromal chaperonin Cpn60 is involved in the final steps of protein translocation into chloroplasts.

Nuclear-encoded proteins of the thylakoid membrane and the thylakoid lumen contain additional thylakoid transfer signals for which degenerate consensus sequences have been discerned (Meadows et al. 1992; Robinson and Klösgen 1994). The splitting off of the transit sequences is effected by a lumenal thylakoid processing peptidase. Apparently, there are three different mechanisms for protein import into the thylakoid lumen and accordingly also three diverse translocation complexes in the thylakoid membrane (Michl et al. 1994): (1) The transport of the 23- and 17-kDa proteins of the oxygen-evolving complex is independent of ATP; however, it depends on a transthylakoidal  $\Delta\text{pH}$ ; (2) the import of (e.g.) plastocyanin is independent of  $\Delta\text{pH}$ , yet it affords ATP and a stromal factor which has turned out to be the chloroplast homologue of bacterial SecA (CPSecA; Yuan et al. 1994); (3) the membrane-integral component  $C_6\text{FII}$  of the thylakoid ATP-synthase complex is imported by a third mechanism which makes use of neither nucleoside triphosphates nor stromal factors and is only slightly stimulated by  $\Delta\text{pH}$ .

The plastid envelope is not only the site of protein import but also a major site of lipid synthesis in plant cells as well as for specific transport of a plethora of metabolites (cf. Douce and Joyard 1990; Flügge and Heldt 1991; Flügge 1997). As in mitochondria, the *com* is equipped with porins and is accordingly highly permeable (Fischer et al. 1994). The *cim* is fitted with a dimeric triosephosphate/phosphate translocator (TPT) functioning as an antiporter. Each of its subunits possesses six transmembrane domains (Heldt and Flügge 1992). The presequence of the TPT contains a stromal targeting information; the incorporation of the TPT into the *cim* is conveyed by a hydrophobic region in the sequence of the mature protein (Knight and Gray 1995).

## 11. Phylogeny of Compartments

As long as cellular life has existed, membranes most probably arose from membranes and compartments from compartments (see Sect. 2). Therefore, the question of how the diversity of eucytic compartments came about is intriguing, not only in the context of cell differentiation but also in connection with phyletic cell evolution.

How did eukaryotes originate with their characteristic cells comprising endomembranes, cytoskeleton, linear chromosomes, and DNA-containing organelles? In view of the extremely scanty microfossil record, possible answers have been sought by comparing RNA and protein sequences on the basis of the "three-domains concept" (Archaea, Bacteria, Eucarya) put forward by Carl Woese and Otto Kandler (cf. Woese et al. 1990) which appears to be impressively supported by the recently published complete genome sequence of the archaeon *Methanococcus jannaschii* (Bult et al. 1996; cf. also Gray 1996). During recent years, data have accumulated indicating a closer phyletic relationship between Archaea and Eucarya (cf. Klenk and Doolittle 1994; on earlier data see Gogarten et al. 1989; on Archaea cf. Kandler 1993). It has, therefore, been speculated that the last common ancestor of the Archaea and the Eucarya would date back less far than the "cenancestor" of all present life. However, not all sequence data fit into such a concept; nor can the general phyletic tree be rooted (Iwabe et al. 1989) without considerable uncertainties (Sogin 1991). Hence, it has been proposed more than once that the protoeukaryotes could have had a chimeric origin in that they came about through fusion between archaean and Gram-negative (eu)bacterial cells (Gupta and Singh 1994; Irwin 1994; Lake and Rivera 1994; see, however, also Roger and Brown 1996). In discussing the earliest evolution of cells and compartments it must be kept in mind that sequence comparisons can tell only little about processes that (presumably) took place in an unimaginable distant past. To mention just one point, it is very difficult, if not impossible, to assess blurring influences of horizontal gene transfer upon reconstructions of the basal region of a general phyletic tree. In fact, the scanty knowledge about the most primitive forms of cellular life permit even very unconventional imagination (for a readable survey of this topic cf. Doolittle and Brown 1994). Not even the generally believed monophyletic origin of earthly life can be taken for granted (Kandler 1994, 1995).

The internal membranes and compartments of primitive eucytes presumably arose partly from invaginations of the PM (forming exoplasmatic compartments with a single membrane; De Duve 1990) and partly by endocytobiosis (in this case, plasmatic compartments arise that are separated from the surrounding cytoplasm by two envelope membranes which never fuse). That mitochondria and plastids go back phylogenetically to once free-living proteo- and cyanobacteria is the basic tenet of the "endosymbiont theory", which is by now well established (cf. Doolittle 1980; Gray 1992; Sitte and Eschbach 1992; Sitte 1993; Maier et al. 1996; Melkonian 1996) and is further supported by continuously growing evidence (e.g., Yuan et al. 1994; Osteryoung and Vierling 1995). Also, the phyletic origin of "complex" plastids by secondary endocytobiosis in algal groups with more than two enveloping membranes around their plastids (eukaryotic, phototroph cytosymbionts are incorporated into



organotrophic host cell: Gibbs 1981; cf. Sitte and Maier 1992; Sitte 1993; McFadden and Gilson 1995; Maier et al. 1996; Melkonian 1996; Palmer and Delwiche 1996) seems to be well established by studies in cryptomonads (McFadden 1990; Douglas et al. 1991; Maier et al. 1991; Cavalier-Smith et al. 1996) and chlorarachniophytes (McFadden et al. 1994, 1995; Gilson and McFadden 1995, 1996; Van de Peer et al. 1996). These two groups are characterized by vestigial nuclei ("nucleomorphs") of eukaryotic cytosymbionts with rudimentary, very small genomes so that sequence comparisons could be carried out for rRNAs. Just why in these taxa, contrary to chromophytes, haptophytes, euglenophytes, and dinoflagellates, nucleomorphs have been retained has been discussed by Häuber et al. (1994). The more general question as to why the common and very massive gene transfer from cytosymbionts to host cell nuclei did not lead to complete loss of organellar genomes can currently only be answered tentatively.

Quite recently it has been shown that hydrogenosomes contain mitochondria-specific heat-shock proteins (Hsp10, Hsp60, Hsp70). Accordingly, these organelles which possess two enveloping membranes but lack cytochromes, the tricarboxylic acid cycle, and DNA are nevertheless likely to represent highly modified mitochondria (cf. Palmer 1997). A renewed discussion on the phyletic origin of peroxisomes by endocytobiosis (Cavalier-Smith 1989) seems desirable in view of these results.

## 12. "Nonplasmatic" Compartments Inside the Cell

After endomembranes had been discovered in eucytes with the electron microscope (EM) it was supposed at first that these membranes would enclose special sorts of cell plasm. Accordingly, new terms were coined (e.g., "Golgi plasm", or "Garnier plasm" for the content of ER cisternae: Ruska 1962). Schnepf (1964, 1966), however, after having studied *Geosiphon pyriforme*, a phycomycete with endosymbiotic *Nostoc* (cf. Kluge et al. 1993), and *Glaucocystis* species, arrived at a radically different interpretation of cell compartmentation. According to Schnepf, not only vacuoles but also the content of ER and Golgi cisternae, as well as the intermembrane spaces of mitochondrial and plastid envelopes and the thylakoid lumen are to be regarded as "wässrige Mischphasen" (today called nonplasmatic or exoplasmatic compartments). "Plasmatic" compartments are restricted to the cytoplasm *sensu stricto* and the nuclear content as well as to the mitochondrial matrix and the plastid stroma. In generalization of the situation at the PM and the tonoplast, Schnepf postulated that every biomembrane would separate "plasm" from "nonplasm". Meanwhile, this concept is generally agreed upon. It has entered many textbooks as the "compartmentation rule" or "Schnepf's theorem". Its consequences appear essential for a profound understand-

ing of cell compartmentation (cf. Sitte 1977, 1980, 1983) and permit, at the same time, critical evaluation of the concept (Schnepf 1984). Some more prominent conclusions are as follows.

- Separation of plasmatic compartments from other plasmatic ones cannot be effected by a single biomembrane but only by at least two membranes, that is, by interposition of a nonplasmatic compartment. Regarding exoplasmatic compartments, the situation is analogous. Accordingly, the (plasmatic) inner compartment of mitochondria as well as the plastidal stroma are separated from the cytoplasm by a double membrane, and the Golgi cisternae of a dictyosome are separated from each other by layers of cytoplasm. If compartments of the same kind come into close contact, as in the grana stacks of chloroplasts, a minimal distance of the tightly appressed membranes is nevertheless maintained by stacking proteins.
- Permeants are translocated from plasmatic to exoplasmatic compartments or *vice versa* by membrane transport. Conversely, for vesicular traffic, translocations are brought about among compartments of the same kind.
- Direct communication is possible only between compartments of the same kind since only then can the limiting membranes fuse.
- Consequently, all exoplasmatic compartments of a given cell should be able to merge and the same should apply to the diverse plasmatic compartments. However, mitochondrial plasm ("mitoplasm") and plastidal stroma ("plastoplasm") never merge with each other nor with the cytoplasm. Correspondingly, not all cellular membranes can fuse (Table 3). The traditional classification of plasmatic compartments as either cytoplasmic or karyo-(nucleo-)plasmic is no longer adequate. All nonfusing plasmatic compartments possess inherent complements of DNAs and RNAs, a fact explained by the endosymbiont theory (see Sect. 11).

**Table 3.** Classes of cellular membranes. Membranes belonging to same "class" may fuse or be derived from each other. Membranes not belonging to same class never come into direct contact with each other

	Characteristic lipid constituents
Plasma membrane, tonoplast, ER and Golgi membranes, mitochondrial and plastid outer membranes, CV, and other vesicle membranes	Steroids
Plastid inner membranes, thylakoids	Trans-3-hexadecenoic acid; plant galactolipids
Mitochondrial inner membranes	Cardiolipin
Peroxisome membrane	-

**Table 4.** Comparison of plasmatic and exoplasmatic compartments

	Plasmatic	Extraplasmatic
DNA, RNA, ribosomes	+	–
Nucleoside triphosphates, phosphoenolpyruvate, NADH, NADPH	+	–
Cytoskeleton and motor molecules	+	–
pH <sup>a</sup>	7.2–7.6	< 6.5
Calcium concentration	Micromolar	> Millimolar
Catabolism of macromolecules	Phosphorolysis	Hydrolysis
Storage polysaccharides: $\alpha$ -glucans (e.g., starch)	+	–
Structural polysaccharides: $\beta$ -glucans (e.g., cellulose)	–	+
Protein glycosylation <sup>b</sup>	Simple, if at all	Complex

<sup>a</sup> On the respective redox conditions, see Section 8a.

<sup>b</sup> Cf. Hart (1992).

- If it is the basic function of any biomembrane to separate plasmatic from exoplasmatic compartments, then the asymmetries of biomembranes become comprehensible. Integral membrane proteins are glycosylated exclusively on their exoplasmatic domains, whereas phosphorylation is confined to their plasmatic domains. H<sup>+</sup>-ATPases always translocate protons from plasmatic into exoplasmatic compartments (or, at the PM, into the external medium). In freeze fracture and freeze etch preparations, corresponding faces of membranes differ from each other by the frequency of inner membrane particles (IMPs) which show a higher density at plasmatic faces (PF) as compared with exoplasmatic ones (EF). When the modern freeze etch nomenclature was introduced by Branton et al. in 1975 the statements of Schnepf were (unconsciously) taken for granted.
- The distinction of plasmatic and exoplasmatic compartments was primarily founded on fine-structural results. Meanwhile, however, this distinction has been amply corroborated by biochemical evidence (Table 4).

### 13. Concluding Remarks and Outlook

The study of transport phenomena at the boundaries of cellular compartments has furnished many details on the translocation of ions, metabolites, signalling compounds, proteins, and nucleic acids. An impressive multitude of transport devices has been discovered, from specific

translocators and membrane channels to complexes composed of different subunits, mediating the transmembrane transport of macromolecules, up the connections between compartments and cells of EM or even light microscopic dimensions (see Sects. 5, 9). Furthermore, specific and unidirectional mass transport as is common in eucytes could be elucidated in appreciable depth. By now, we are fairly well informed about what material may be translocated by a particular membrane or vesicle species. However, it would likewise be important to know for certain what *cannot* be translocated at a given membrane and which reactions accordingly will *not* take place in the corresponding compartment. Furthermore, most of the published results are of a qualitative rather than a quantitative nature. A general view of cell compartmentation has, however, to rely on quantitative data (as provided, e.g., by Steer 1981; Winter et al. 1993, 1994; Leidreiter et al. 1995 a,b) that eventually could lay the basis for mathematical cell models. Only then could the functional relationships between the different compartments in the cellular microcosm be firmly established in all necessary details. It is beyond doubt that the genesis and activity of the diverse cellular compartments must be perfectly coordinated (Shyjan and Butow 1993; Herrmann 1996; Nunnari and Walter 1996). The signals for intracellular dialogue are, however, still largely unknown. Concerning the genesis and dynamics of compartments, many interesting details have been discovered. Yet a comparison with the review on this topic as presented by Morré in 1975 makes it clear that some basic questions still remain unanswered.

The functional interactions between compartments and cytoskeletal elements are insufficiently known as yet. It can be expected, however, that new insights regarding these interactions are imminent, since the exploration of the cytoskeleton has recently made dramatic progress.

In spite of all peculiarities of the great (and partly extremely diverse) organismal phyla, fundamental conformity dominates in the domains of cell and molecular biology, pointing to a monophyletic origin of all eukaryotes. This fact (which possibly justifies the quotation of so many papers out of the realm of medical and animal sciences in this chapter) permits the establishment of common principles (see Wickner 1994; Schatz and Dobberstein 1996). This should in turn facilitate the analysis of compartments in plant and algal cells, based on the work already successfully done with particularly suitable model systems such as yeasts, mammalian cultured cells, or amphibian oocytes. Still, the investigation of compartments typical for plant cells (plastids, vacuoles, and cell walls) will of course be particularly rewarding.

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## **Genetics**

## Recombination: Molecular Markers for Resistance Genes in Major Grain Crops

By Frank Ordon, Willy Wenzel, and Wolfgang Friedt

### 1. Introduction

Grain crops, i.e. rice (*Oryza sativa*), maize (*Zea mays*), wheat (*Triticum aestivum*), barley (*Hordeum vulgare*), and to a lesser extent sorghum (*Sorghum bicolor*), oats (*Avena sativa*), and rye (*Secale cereale*) are of major importance for human nutrition (cf. Anonymous 1994). However, each year sincere yield losses have to be faced in these crops due to fungal and viral diseases as well as insect pests. Therefore, breeding for resistance in order to avoid these losses as well as the application of fungicides and pesticides has to be considered as a major goal in breeding of these cereals. In general, combining of resistances or the introgression of new resistance genes from unadapted germplasms or related species, respectively, is achieved by sexual recombination, i.e. crossing of parental lines followed by phenotypic selection in the segregating offspring. In this case, the success of breeding entirely depends on extensive field or glasshouse tests for resistance to the respective pathogens. However, as grain crops are damaged by many pathogens which often show a rapid adaptation to their hosts' resistance genes, breeding for resistance is a very complex task and the identification of desired recombinants expressing resistance to most diseases by phenotypic selection only has nearly reached the limits of manageability.

However, methods of plant biotechnology like the use of anther and microspore techniques giving rise to homozygous doubled haploid plants in  $A_1$  ( $F_2$ ) already, thereby facilitating a more rapid and easier phenotypic selection (cf. Devaux et al. 1996; Kasha 1996), and especially the development of molecular marker systems transferring selection to some extent from the phenotypic to the genotypic level, offer new opportunities for a more efficient breeding for resistance today (cf. Graner 1996; Langridge et al. 1996).

As the success of marker-based selection procedures entirely depends on the availability of markers tightly linked to the respective resistance gene(s) the development of molecular marker systems starting from restriction fragment length polymorphisms (RFLPs; Botstein et al. 1980), via the application of polymerase chain reaction (PCR)-based proce-

**Table 1.** Characteristics of molecular marker systems (According to Powell et al. 1996, modified)

	RFLP	RAPD	SSR	AFLP
Principle of assay	Endonuclease digestion and hybridization	Amplification with random primers	Amplification of SSRs	Amplification of DNA fragments limited by random nucleotides
Type of polymorphism detected	Single base insertions or deletions	Single base insertions or deletions	Repeat length	Single base insertions or deletions
Dominance	Co-dominant	Dominant	Co-dominant	Dominant
Amount of DNA required	2–10 µg	10–25 ng	25–50 ng	0.2–0.5 µg
DNA sequence required	No	No	Yes	No

dures (Saiki et al. 1985, 1988), e.g. the use of random amplified polymorphic DNAs (RAPDs; Welsh and McClelland 1990; Williams et al. 1990), the detection of simple sequence repeat polymorphisms [microsatellites, simple sequence repeats (SSRs), Tautz and Renz 1984; Röder et al. 1995], the use of random amplified microsatellite polymorphisms (RAMPs; Wu et al. 1994), and the application of amplified fragment length polymorphisms (AFLPs; Zabeau and Vos 1993; Vos et al. 1995) have dramatically increased the possibilities of an efficient application of marker-based selection procedures in plant breeding. Major characteristics of some of these techniques widely used in plant breeding today, which will not be explained in detail in this chapter, are summarized in Table 1.

These molecular techniques have gained evident importance in plant breeding today, e.g., in accelerated back-crossing procedures (e.g. Powell et al. 1996; Tanksley and Nelson 1996), in 'pyramiding' resistance genes (e.g. Pedersen and Leath 1988; Inukai et al. 1996), in the analysis of and selection for quantitative traits, e.g. in barley (e.g. Thomas et al. 1995; Oziel et al. 1996; Tinker et al. 1996) or in the varietal identification and estimation of genetic relationships in wheat and its relatives (e.g. Vierling and Nguyen 1992; Castagna et al. 1994; Siedler et al. 1994), barley (e.g. Graner et al. 1994; Melchinger et al. 1994; Ordon et al. 1997), oats (e.g. Goffreda et al. 1992; Heun et al. 1994; Moser and Lee 1994; O'Donoghue et al. 1994), rye (e.g. Iqbal and Rayburn 1994; Loarce et al. 1996a), rice (e.g. Wang and Tanksley 1989; Zhang et al. 1992; Virk et al. 1995), sorghum (e.g. Aldrich and Doebley 1992; Cui et al. 1995; DeOliveira et al. 1996), and especially in maize (e.g. Melchinger et al. 1991; Bernardo 1993) where heterotic groups have been established based on these data (e.g. Lee et al. 1989; Dudley et al. 1991; Melchinger et al. 1992; Messmer et al. 1993; Dubreuil et al. 1996). However, one of the most important applications of molecular markers in breeding major grain crops is the use in marker-assisted selection (MAS) procedures for disease resistance which this chapter will bring into focus.

## 2. Marker-Based Selection – Some Case Histories

Historically the concept of using markers originated with the recognition that genes do not always follow Mendel's law of independent assortment, and that genes can be assigned to linkage groups; i.e. genes are linearly arranged on chromosomes and the distance between the genes can be estimated by the relative recombination frequency. An overview on the different marker classes and methods of estimating recombination frequencies in different population types is given by Weber and Wricke (1994). In a first step, easily detectable morphological traits giving a clear-cut phenotype have been mapped, e.g. the genes for hooded lemma (*K*), orange lemma (*o*) or naked karyopsis (*n*) in barley (cf. Tsuchiya 1982). Mapping of these genes on the respective chromosomes has been aided by chromosomal aberrations or chromosome addition lines, e.g. in wheat and barley (Tsuchiya 1967; Sears 1972). For example, the barley yellow mosaic virus resistance gene *ym4* has been located on the long arm of barley chromosome 3 by using aneuploid lines, i.e. trisomics and telotrisomics (Kaiser and Friedt 1989, 1992). However, only a limited number of easily detectable morphological marker genes are available in grain crops, e.g. in barley (cf. Søgaard and von Wettstein-Knowles 1987) which in general represent undesirable agronomic traits and are therefore not present in adapted plant breeding populations. Consequently, the use of these marker types in practical plant breeding is rather limited and the occurrence of a close linkage between a desirable agronomic trait, like the resistance gene *ym1* and the gene *K* for hooded lemma in barley, has to be considered as a rather rare event (Takahashi et al. 1973; Konishi et al. 1997).

The period of morphological markers mainly analysed by cytogenetic methods – which, however, are still a prerequisite for the construction of genetic linkage maps – was followed by the application of isozyme analysis (Markert and Møller 1959), e.g. in barley (Hvid and Nielsen 1977; Nielsen and Johansen 1986), wheat (Hart et al. 1980), maize (Heidrich-Sobrinho and Cordeiro 1975; Frei et al. 1986), oats (Almgard and Clapham 1975; Phillips and Murhpy 1993) or rye (Wehling et al. 1985; Melz et al. 1992; Benito et al. 1994). An overview on the different aspects of the use of isozyme electrophoresis in plant breeding is given by Tanksley and Orton (1983).

Using these techniques, e.g. linkage between resistance genes (*ym4*, *ym5* and *ym6*) to barley yellow mosaic viruses and the esterase isozyme cluster *Est1-Est2-Est4* on the long arm of chromosome 3 of barley (Konishi et al. 1989, 1997; Konishi and Kaiser 1991; Iida and Konishi 1994; Le Gouis et al. 1995) or linkage between BaYMV-resistance introgressed from *Hordeum bulbosum* and a peroxidase locus have been detected (Zhang et al. 1995). Furthermore, linkage between the endopeptidase *EP-1* on wheat chromosome 7D and the eyespot resistance gene *Pch-1* from *Aegilops ventricosa* (McMillin et al. 1986; Vahl and Müller 1991) or a close linkage between a null allele of the endopeptidase *Ep-D1c* and the leaf rust resistance gene *Lr19* have been found (Winzeler et al. 1995).



Besides the fact that the isozyme technology can be employed with relatively low cost, isozymes – opposite to morphological markers – are in general inherited in a codominant fashion (McMillin and Allan 1987), independent of the environment (Arus et al. 1982) and free of epistatic effects (Tanksley 1983) and can be considered as a nearly ideal marker system, therefore. Consequently, isozyme marker maps have been constructed for many crops (Stuber 1992) and isozyme analysis has been widely used in plant breeding. However, due to the limited number of isozyme systems available in plant species and the varying degree of polymorphisms, isozymes – like morphological markers – gain only poor genomic coverage. Consequently, the period of isozyme electrophoresis has been followed by the application of marker systems detecting polymorphism directly on the DNA-level, like RFLPs (e.g. Beckmann and Solter 1986; Bernatzky and Tanksley 1986) and the above mentioned PCR-based molecular techniques (Table 1).

### 3. Construction of Molecular Linkage Maps

With the rise of these molecular techniques and the development of the respective computer software (e.g. Lander et al. 1987) linkage maps being a prerequisite to locate resistance genes on specific chromosomes have been constructed for all major grain crop species.

In maize ( $2n = 2x = 20$ ),  $F_2$  populations and recombinant inbred lines (RILs) have been used for this purpose (cf. Helentjaris et al. 1986, 1988; Burr et al. 1988; Beavis and Grant 1991; Chao et al. 1994) resulting in the construction of extensive molecular maps. First attempts to construct a sorghum linkage map aimed at resolving the phylogenetic relationship between sorghum ( $2n = 2x = 20$ ) and maize. Therefore, mainly maize probes have been used (Hulbert et al. 1990; Binelli et al. 1992; Whitkus et al. 1992; Melake Berhan et al. 1993) and sorghum derived probes have been added in a second stage of map development (Pereira et al. 1993; Ragab et al. 1994). Following the availability of sorghum probes, dense maps equal to the chromosome number have been established (Chittenden et al. 1994; Xu et al. 1994), and recently polymorphic SSRs have been identified in sorghum suitable to be incorporated in the existing map (Brown et al. 1996). In rice ( $2n = 2x = 24$ ) a first RFLP map was constructed by McCouch et al. (1988) using an  $F_2$ -population derived from a cross between an indica and a javanica cultivar. This map consisted of 135 loci which have been assigned to the 12 rice chromosomes with the help of primary trisomics. In the following time the number of markers located on the rice genome has steadily been increased (Saito et al. 1991; Causse et al. 1994) leading to very dense maps (Kurata et al. 1994). Instead of  $F_2$ -populations the map of Huang et al. (1994) is based on doubled haploid lines being well suited to incorporate RAPD markers in general following a dominant mode of inheritance.

In barley ( $2n = 2x = 14$ ) first full linkage maps based on doubled haploid lines have been developed by Graner et al. (1991), Heun et al. (1991) and Kleinhofs et al. (1993). Out of these maps a joint map has been constructed using a cross between *Hordeum spontaneum* and *H. vulgare* (Sherman et al. 1995). Besides these primarily RFLP-based maps, a RAPD map of barley has been established by Giese et al. (1994), and recently SSRs have been integrated in the different RFLP maps (Liu et al. 1996). Since all the barley linkage

maps are built on doubled haploid lines they provide an ideal resource for integrating AFLP markers. Becker et al. (1995) have added 118 AFLP markers to the 'Proctor' 'Nudinka' population and Powell et al. (1996) have assigned between 202 and 580 markers to different mapping populations. Therefore, different density maps of the barley genome are available today being an ideal base for marker-based selection.

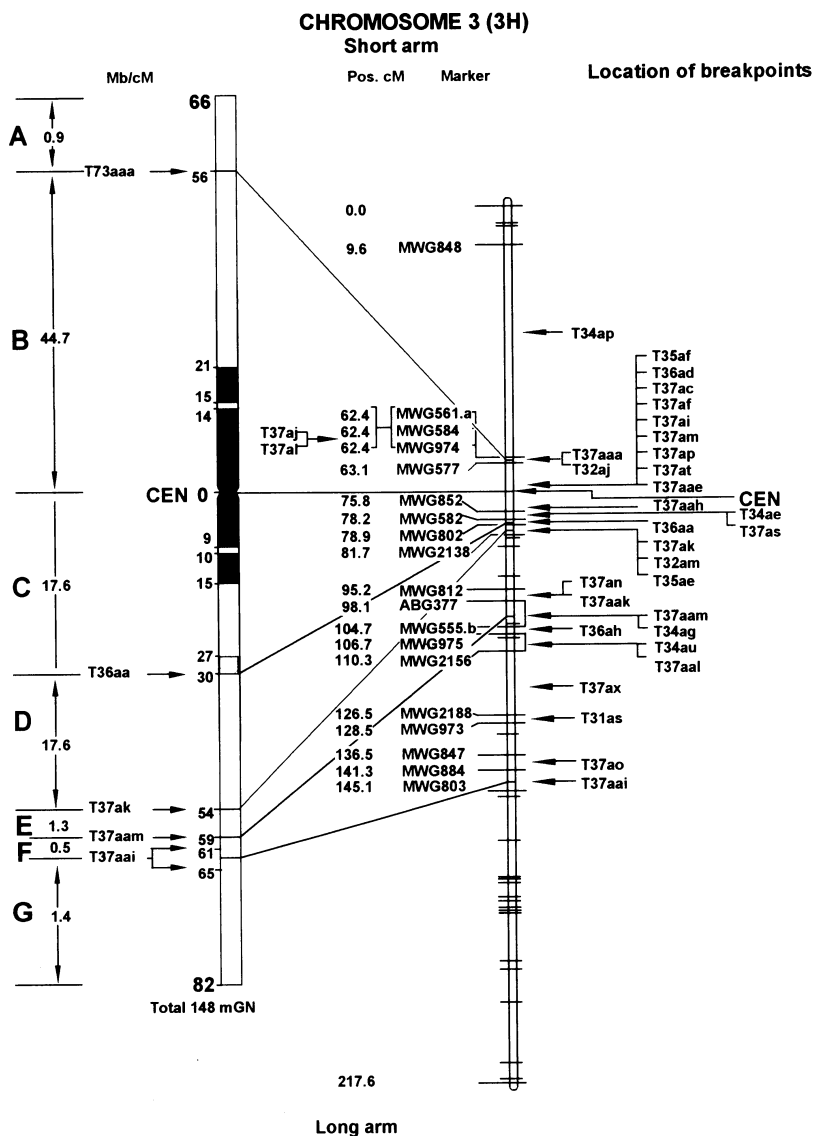
In comparison to the diploid species mentioned above, the linkage map of rye ( $2n = 2x = 14$ ) consisting of RFLPs, RAPDs and physiological markers (Wricke 1991; Philipp et al. 1994) has to be considered as not very dense. More extensive linkage maps are available for single chromosomes due to comparative mapping to other grain crops (Wang et al. 1992; Devos et al. 1993; Wanous and Gustafson 1995). However, recently a linkage map of six rye chromosomes consisting of 77 RFLP and 12 RAPD markers has been developed by Loarce et al. (1996b).

The construction of genetic linkage maps in polyploid species, like common wheat ( $2n = 6x = 42$ ), is more difficult in comparison to diploid species. However, full linkage maps of the wheat genome as well as RFLP maps of the different homoeologous groups providing a high genetic resolution have been constructed (e.g. Liu and Tsunewaki 1991; Devos and Gale 1993; Devos et al. 1993; F.Q. Chen et al. 1994; Nelson et al. 1995 a,b) and SSRs have been assigned to the different chromosomes of wheat already (Röder et al. 1995).

Cultivated oats (*Avena sativa*,  $2n = 6x = 42$ ) is a hexaploid species, too. First linkage maps were constructed on diploid species (O'Donoughue et al. 1992; Rayapati et al. 1994), e.g. on a cross between *A. atlantica* and *A. hirtula* in which 192 marker loci have been assigned to the seven chromosomes of the A genome (O'Donoughue et al. 1992). Meanwhile, a full linkage map of cultivated oats is available (O'Donoughue et al. 1995).

The construction of molecular linkage maps which was mainly based on RFLPs in a first step and has consequently lead to more dense maps by the application of PCR-based techniques has added a new dimension to resistance breeding in grain crops, namely the possibility to locate any resistance gene of interest on a specific genomic region. This enables the plant breeder to calculate the possibility of combining different resistance genes by recombination and to follow the process of recombination by using the respective markers. Besides the chromosomal location of different genes these maps are an ideal base for the construction of high density maps around the loci of interest (DeScenzo et al. 1994; Mahadevappa et al. 1994; Bauer and Graner 1995) being a prerequisite for map-based cloning strategies in complex genomes (Tanksley et al. 1995).

However, it always has to be taken into account that recombination frequencies are not constant along chromosomes leading to a non-linear relationship between genetic and physical distances. Today, corresponding relationships in different chromosomal regions have been partially estimated by the use of molecular maps and cytogenetic techniques, e.g. in wheat by so-called cytogenetic laddermaps (Mickelson-Young et al. 1995) and in barley by the use of reciprocal translocation lines (Künzel and Korzun 1996). As an example, the relationship between physical and genetic distances for barley chromosome (3H) is shown in Fig. 1. Suppressed recombination areas in the proximal regions represent 74% of



**Fig. 1.** Comparison between physical and genetic map of barley chromosome 3 (3H) (Künzel and Korzun 1996)

the total length of this chromosome [148 milliGeNomes (mGN)] and the correspondence between physical and genetic maps of defined subregions varies between 0.5 and 44.7 Mb/cM (megabases/centiMorgan). Furthermore, it turned out that in the distal part of the long arm of chromosome 3 a hotspot of recombination is present. These facts have to be taken into account when estimating the possibilities of a positional cloning approach, e.g. of respective resistance genes incorporated in genetic linkage maps.

#### 4. Application of DNA Polymorphisms in Breeding for Resistance

The application of markers for tagging of and selection for resistance genes in major grain crops has progressed very rapidly in the past 5 years. At first, most emphasis has been given to the detection of markers for qualitative resistance genes, but as map development and statistical programmes emerged more quantitative trait loci (QTLs) for disease resistances have been mapped. However, independent from the mode of inheritance of resistance the use of selectable markers offers the opportunity of a more efficient management of resistance genes during the process of recombination and selection. The present chapter will give an overview on genes already tagged by molecular markers in grain crops facilitating MAS.

##### a) Maize and Sorghum

In comparison to wheat or barley, maize is attacked by a limited number of fungal and viral pathogens only. An overview on the genomic organization of disease and insect resistance in maize is given by McMullen and Simcox (1995). In the northern USA corn belt and other temperate regions northern leaf blight caused by *Helminthosporium turcicum* (syn. *Exserohilum turcicum*) is an important foliar disease. By using RFLP analysis of near isogenic lines the major resistance gene *Ht1* has been mapped on chromosome 2L (Bentolila et al. 1991), and the genes *Ht2* (Zaitlin et al. 1992) and *HtN* have been assigned to chromosome 8L (Simcox and Bennetzen 1993). Besides these resistance genes additional QTLs for the components of disease development have been detected by using unselected F<sub>2</sub> and F<sub>3</sub> lines (Freyemark et al. 1993, 1994).

Furthermore, reaction to *Puccinia sorghi* which is the causal agent of a rust disease in maize has been mapped. Resistance to this disease is conferred by different dominant *Rp*-loci showing multiple allelism (Wilkinson and Hooker 1968), or in the case of *Rp1* represents a series of closely linked genes with different specificities (Hulbert and Bennetzen 1991; Hu and Hulbert 1994, 1996). Closely linked RFLP markers permit-

ting marker-assisted entry of *Rp3* in different maize backgrounds have been detected by Sanz-Alferez et al. (1995), and it has been shown that concerning the *Rp1*-locus flanking marker exchange due to crossing over within this gene complex is associated with new rust resistance specificities (Richter et al. 1995; Hu and Hulbert 1996).

Besides these fungal pathogens *Colletotrichum graminicola* causing anthracnose stalk rot in maize is of some importance. RFLP-based quantitative analysis indicated a significant QTL on linkage group 4 in both populations tested in this study (Jung et al. 1995).

Concerning resistance to insects, seven QTLs explaining 38% of the phenotypic variation of resistance to the second generation of the European corn borer (*Ostrinia nubilalis*) have been detected by using the tunnel length as a criterion of resistance (Schön et al. 1993). Besides these pathogens maize is hit by the aphid-transmitted potyvirus maize dwarf mosaic virus (MDMV) and some inbred lines are susceptible to the mite-transmitted potyvirus wheat streak mosaic virus (WSMV). Concerning the *mdm1* locus conferring resistance to five strains of MDMV it has been shown by morphological and RFLP analysis that it is located on the short arm of chromosome 6 and cosegregating with the nucleolus organizer region (NOR) in maize (Simcox et al. 1995). By using bulked segregant analysis (Michelmore et al. 1991) to detect linkage to RFLP loci three genes conferring resistance to WSMV have been localized, i.e. *wsm1* on chromosome 6, *wsm2* on chromosome 3 and *wsm3* on chromosome 10. In this case it is interesting to note that severe mosaic is only found in the case of homozygous alleles of the susceptible parent at all three loci (McMullen et al. 1994).

In sorghum, which like maize belongs to the tribe *Andropogoneae*, RFLP and RAPD marker for resistance genes to head smut (*Sporisorium reilianum*), acremonium wilt (*Acremonium strictum*) and downy mildew (*Peronospora sorghi*) have been developed (Oh et al. 1992, 1993).

## b) Rice

Rice is the staple food for about two-thirds of the world's population and about 90% of the world's rice is grown and consumed in developing countries. Therefore, breeding of resistant varieties is of special importance in this crop. One of the most serious diseases of rice worldwide is rice blast caused by *Pyricularia grisea* (syn. *Magnaporthe grisea*). At least 12 dominant genes conferring complete resistance and 10 QTLs associated with partial resistance have been mapped already (McCouch et al. 1994, cited in Yu et al. 1996). By using nearly isogenic lines (NILs) and RFLP analysis the gene *Pi-2(t)* has been located on chromosome 6 and gene *Pi-4(t)* on chromosome 12 of rice, respectively (Yu et al. 1991). Furthermore, genes *Pi-5(t)* and *Pi-7(t)* as well as ten QTLs affecting resis-

tance have been mapped using RILs (Wang et al. 1994; Inukai et al. 1996), and in additional studies the gene *Pi-1(t)* has been assigned to chromosome 11 and another gene which may be allelic to *Pi-4(t)* to chromosome 12 (Yu et al. 1996). By applying a PCR-based approach a sequence characterized amplified region (SCAR) being tightly linked to the resistance gene *Pi-10* (Naqvi and Chattoo 1996), and a specific amplicon region (SAP) linked to *Pi-2(t)* (Hittalmani et al. 1995) have been identified as being well-suited for practical plant breeding purposes.

Besides rice blast, bacterial blight caused by *Xanthomonas oryzae* pv. *oryzae* is of major importance in rice breeding. Different genes conferring resistance to bacterial blight have been identified by analysing the genetic basis of host resistance with some of them mapped on different chromosomes, i.e. the dominant genes *Xa-1*, *Xa-3*, *Xa-4* (Yoshimura et al. 1992) and *Xa-21* (Ronald et al. 1992; Williams et al. 1996) as well as the recessive genes *xa-5* (McCouch et al. 1991) and *xa-13*, respectively (Zhang et al. 1996). Concerning the gene *Xa-1* a yeast artificial chromosome (YAC) carrying this gene has been identified recently, being the first step to positional cloning and functional analysis of the respective resistance gene (Yoshimura et al. 1996).

With respect to *Rhizoctonia solani*, the causal agent of sheath blight, no major genes giving complete resistance have been identified so far. However, considerable variation is present within rice varieties for quantitatively inherited resistance: six QTLs explaining 47% of the phenotypic variation have been detected by RFLP analysis (Zhikang et al. 1995).

Besides fungal and bacterial diseases, insects cause severe yield losses in rice. In this context the gall midge (*Orseolia oryzae*) is of major importance. PCR-based DNA markers tightly linked to the resistance genes *Gm2* and *Gm4t* facilitating efficient marker-based selection procedures independent of the availability of insects have been developed, enhancing the progress of breeding resistant varieties (Mohan et al. 1994; Nair et al. 1995, 1996). Furthermore, dominant resistance to the brown planthopper has been mapped on chromosome 12 (Ishii et al. 1994; Hirabayashi and Ogawa 1995), and resistance to the green leafhopper (*Nephotettix virescens*) vectoring rice tungro spherical virus (RTSV) and rice tungro bacilliform virus (RTBV) has been located on chromosome 4 (Sebastian et al. 1996). In this respect, it is interesting to note that resistance to the green leafhopper is cosegregating with resistance to RTSV but is independent of RTBV resistance. The question whether resistance to the green leafhopper and RTSV is governed by two linked genes or is the result of pleiotrophic action of a single locus is still under investigation.

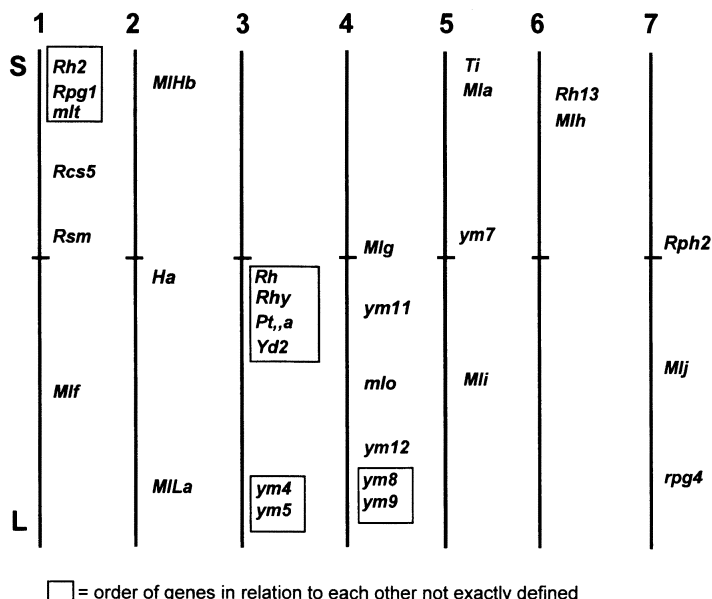


Fig. 2. Schematic arrangement of major resistance genes of barley tagged by molecular markers (for abbreviations of genes see text). (According to Graner 1996; Graner pers. comm.)

### c) Barley

Owing to the fact that barley is grown from the polar circle to the tropics it is hit by a wide range of different fungal and viral diseases and many resistance genes have been tagged already (Fig. 2; for review see Graner 1996).

In this respect, powdery mildew caused by *Erysiphe graminis* has to be considered as a particularly serious disease. Resistance to this disease is governed by different major race-specific genes of which some have been tagged and localized by molecular markers. The first one in this respect has been the multiallelic *mlo* locus on chromosome 4L (Hinze et al. 1991). Among the loci conferring resistance to powdery mildew the *mlo* locus has some outstanding features: the resistance alleles are inherited recessively and up until now resistance is effective against all races of the fungus. Besides *mlo* the *Mlg* locus has also been assigned to chromosome 4 (Görg et al. 1993). Another multiallelic locus is the *Mla* locus on chromosome 5S (Schüller et al. 1992). Different alleles of this locus consisting of at least 32 alleles – some of them deriving from *H. spontaneum* – have been detected already by RFLP analysis (Schüller et al. 1992; Jahoor et al. 1993; Kintzios et al. 1995). Besides these loci,

introgressed genes from *H. spontaneum*, i.e. *mlt*, *MLf* and *MLj*, have been localized on chromosome 1S, chromosome 1L and chromosome 7L, respectively (Schönfeld et al. 1996). *MLLa* derived from *H. laevigatum* is located on chromosome 2L (Hilbers et al. 1992; Giese et al. 1993; Mohler and Jahoor 1996) and *MLhb* derived from *H. bulbosum* on chromosome 2S (Pickering et al. 1995; Graner et al. 1996b). An overview on resistance genes introgressed from *H. bulbosum* is given by Pickering et al. (1996). In addition to these major genes, different QTLs for resistance to powdery mildew have been detected (Heun 1992; Saghai Maroof et al. 1994; Backes et al. 1995; Thomas et al. 1995).

Besides powdery mildew, cereal rusts, i.e. *Puccinia graminis*, *Puccinia hordei* and *Puccinia striiformis*, are of worldwide importance in barley production. Concerning *P. graminis*, the resistance genes *RPG1* and *rpg4* have been located on chromosomes 1S and 7L, respectively (Kilian et al. 1994; Borovkova et al. 1995; Horvarth et al. 1995). The resistance gene *Rph2* governing reaction to *P. hordei* has been mapped on chromosome 7 (Steffenson, cit. in Graner 1996) and a RAPD marker linked to a leaf rust resistance gene of the variety Q21861 derived from CIMMYT, Mexiko has been developed (Poulsen et al. 1995). Furthermore, concerning *P. striiformis* two QTLs have been detected (Z. Chen et al. 1994a).

Another important fungal disease of barley is scald caused by *Rhynchosporium secalis*. By using RAPD analysis based on near isogenic lines the gene *Rhy* has been located on chromosome 3L (Barua et al. 1993) and in the following time the gene *Rh2* has been assigned to chromosome 1S (Schweizer et al. 1995), *Rh13* to chromosome 6S (Abbott et al. 1995) and *Rh* to chromosome 3L (Graner and Tekauz 1996). Additional QTLs have been detected on chromosome 3L near the *Est1-Est2* locus, respectively (Thomas et al. 1995).

A major gene conferring resistance to net blotch (*Pyrenophora teres*) in the seedling stage has been located on chromosome 3L close to the *Rh* gene (Graner et al. 1996a) and seven QTLs responsible for resistance at the adult stage accounting for 67.6% of the phenotypic variation and three QTLs for resistance at the seedling stage have been detected recently (Steffenson et al. 1996). Furthermore, it has been shown that resistance to spot blotch (*Cochliobolus sativus*) in the seedling stage is governed by a single gene on chromosome 1S, and that concerning adult resistance 70.1% of the variation is due to two QTLs (Steffenson et al. 1996). In addition to these studies, RAPD markers linked to spot blotch resistance have been detected (Kutcher et al. 1996). Due to the location of resistance to *P. teres* and *C. sativus*, combining high levels of resistance to both diseases should be feasible. Besides these fungal diseases seed-borne barley leaf stripe caused by *Pyrenophora graminea* is of importance. Two QTLs explaining 58.5% and 29.3% of the variance have been detected on chromosomes 1 and 2, respectively (Pecchioni et al. 1996).



Especially in areas with a long snow coverage during winter *Typhula incarnata* has to be considered as an important pathogen of winter barley. Due to its environmental variability, selection for resistance in field tests is very difficult. However, closely linked markers for the gene *Ti* on chromosome 5S have been identified, recently (Graner et al. 1996b).

Besides these fungal diseases barley is hit by some viruses. With respect to economics the aphid-transmitted barley yellow dwarf virus (BYDV) has to be considered as most important. Resistance or tolerance to the different strains of this virus is strongly influenced by environmental factors and the genetic background. The gene (*Yd2*) being responsible for this tolerance has been located in the centromeric region of chromosome 3 by RFLP analysis meanwhile (Collins et al. 1996). Another important disease restricted to Europe and Japan is barley yellow mosaic disease caused by different strains of barley mild mosaic virus (BaMMV) and barley yellow mosaic virus (BaYMV). Different recessive genes conferring resistance to these virus strains are known and have been located on chromosome 3L, i.e. *ym4* (Graner and Bauer 1993; Ordon et al. 1995; Weyen et al. 1996) and *ym5* (Graner et al. 1995), and on chromosome 4L, i.e. *ym8* and *ym9* (Graner et al. 1995), as well as *ym11* and *ym12* (Graner et al. 1996b). Furthermore, the gene *ym7* has been mapped to 5S (Graner et al. 1995). In comparison with these viruses barley stripe mosaic virus (BSMV) is of importance in some areas of North America only. A single gene (*Rsm*) conferring resistance has been mapped on chromosome 1S (Edwards and Steffenson 1996).

In comparison with fungal and viral diseases only a few molecular markers for genes conferring resistance to aphids or nematodes are known, i.e. concerning the Russian wheat aphid (*Diuraphis noxia*; Nieto-Lopez and Blake 1994) and to *Heterodera avenae* (Langridge, cit. Graner 1996). In summary it may be stated that marker data concerning resistance genes in barley in conjunction with the availability of haploid techniques (Deveaux et al. 1996) will facilitate efficient marker-based selection procedures in this crop.

#### d) Wheat

Wheat like barley is affected by many fungal and viral diseases. Due to the allohexaploid nature of wheat ( $2n = 6x = 42$ ) this crop allows more sophisticated procedures of chromosome engineering than diploid species. Therefore, many resistance genes present in common wheat today are derived from crosses with wild relatives and related species. A respective overview is given by Friebe et al. (1996a). Besides the fact that mapping genes in polyploid species is much more difficult than in diploid species, it has to be taken into account that alien introgressions are

normally conserved in linkage blocks around the gene of interest, having reduced recombination.

In wheat grown under temperate climatic conditions powdery mildew caused by *Erysiphe graminis* f. sp. *tritici* is one of the most important foliar diseases. Up until now 22 loci for mildew resistance (*Pm*) have been described. By the use of NILs and nullitetrasonic lines the multiallelic locus *Pm3* has been assigned to homoeologous group 1 (Hartl et al. 1993). Later on, RFLP markers for *Pm2* on chromosome 5D, *Pm1* on chromosome 7A and additional RAPD markers for *Pm18* on chromosome 7A – a gene highly effective against all races present in Europe – have been detected (Hartl et al. 1995). Recently, the following genes have been mapped: *Pm12* derived from *Aegilops speltoides* on chromosome 6B (Jia et al. 1996), *Pm13* derived from *Aegilops longissimum* on 3B and 3D (Donini et al. 1995), *Pm21* derived from *Haynaldia villosa* on chromosome 6 (Qi et al. 1996) and a new gene named *Pm22* derived from cv. 'Virest' on chromosome 1D (Peusha et al. 1996). However, when initiating marker-based selection procedures for powdery mildew resistance it has to be taken into account that some of these genes, like *Pm8* or *Pm17*, may be suppressed by other ones (Ren et al. 1996; Zeller and Hsam 1996).

Besides powdery mildew, leaf rust caused by *Puccinia recondita* is an important foliar disease of common wheat. RFLP and RAPD markers for the *Lr9* leaf rust resistance gene derived from *Aegilops umbellulata* and the *Lr24* gene derived from *Agropyron elongatum* have been developed by Schachermayr et al. (1994, 1995). Since *Lr9* is located on chromosome 6 and *Lr24* on chromosome 3, these genes may be easily combined in order to achieve more durable resistance. Another leaf rust resistance gene introgressed from *Aegilops ventricosa* has been located on chromosome 2A (Bonhomme et al. 1995). Furthermore, the leaf rust resistance locus *Lr1* has been assigned to chromosome 5DS and a sequence tagged site (STS) marker facilitating efficient marker-based selection has been developed (Feuillet et al. 1995). Concerning stem rust caused by *Puccinia graminis* many genes conferring resistance are known. In this respect RFLP markers have been developed for the *Sr22* gene introgressed from *Triticum boeoticum* and it has been shown that the alien chromatin covers a large region of the respective chromosome 7A (Paull et al. 1994). Furthermore, by mapping homoeologous groups 2, 4, 5 and 7 two presumed leaf and stem rust resistance genes have been assigned to group 2 (Nelson et al. 1995a) and *Lr34* has been mapped on 7DS (Nelson et al. 1995b).

Due to limited crop rotation *Pseudocercospora herpotrichoides*, the causal agent of eyespot, has gained evident importance in intensive wheat growing areas. Resistance to this fungus is conferred by the dominant gene *Pch-1*. DNA markers closely linked to this gene located on

chromosome 7D which has been introduced from *Aegilops ventricosa* have been developed by Mena et al. (1992).

Besides the fungal diseases already mentioned, common bunt caused by *Tilletia tritici* and *T. laevis* as well as Karnal bunt caused by *Tilletia indica* are of importance in some areas. With respect to Karnal bunt, a marker-based introgression of resistance derived from barley has been proposed (Blake et al. 1996), and concerning common bunt a RAPD marker for the resistance gene *Bt-10* conferring resistance to most races of the fungus has been identified recently (Demeke et al. 1996).

As in barley, the BYDV causes severe yield losses in wheat too. Resistance to this virus has been introgressed from *Agropyron* (*Thinopyrum*) *intermedium*. By using disomic addition lines ( $2n = 6x + 2 = 44$ ) and translocation lines it has been shown that resistance against the virus is due to genes located on chromosome 7A (Banks et al. 1995; Hohmann et al. 1996) and on homoeologous group 2 (Larkin et al. 1995). Furthermore, it turned out that addition lines carrying an added group 7 chromosome of *Th. intermedium* are resistant to leaf, stem and stripe rust as well (Larkin et al. 1995). Another important virus disease of cultivated bread wheat is wheat streak mosaic virus (WSMV) vectored by the wheat curl mite (*Eriophyes tulipae* syn. *Aceria tulipae*). Resistance to this virus has been introgressed from *Agropyron intermedium* too, and the resistance gene *Wsm1* has been assigned to wheat group 4 chromosomes by RFLP analysis (Friebe et al. 1996b). Furthermore, STS and RAPD markers well suited for transferring *Wsm1* into adapted cultivars have been reported (Talbert et al. 1996).

Resistance to the vector of WSMV, *E. tulipae*, has been introgressed from *Aegilops tauschii* and *Agropyron elongatum*. Recently, it has been shown by using *in situ* hybridization and STS primers that in wheat-*Haynaldia villosa* hybrid lines a gene conferring resistance to mite colonization is located on the short arm of the group 6 chromosome of *H. villosa* (Chen et al. 1996), being an additional source for mite resistance in wheat, therefore. Besides the wheat curl mite the Hessian fly (*Mayetiola destructor*) is a very destructive insect pest of wheat. Following a gene-for-gene relationship, 25 resistance genes have been identified so far. Out of these genes *H23* and *H24* have been assigned to chromosome 6D and 3D by RFLP analysis, respectively (Ma et al. 1993). Furthermore, using NILs a RAPD marker cosegregating with the resistance gene *H9* has been identified (Dweikat et al. 1994). This dominant gene was thought to confer resistance to all known biotypes of the Hessian fly. However, recently a virulent biotype has been isolated (Formusoh et al. 1996). Another economically damaging pest of wheat is the cereal cyst nematode (*Heterodera avenae*). Two flanking RFLP markers facilitating marker-assisted selection for resistance to the cereal cyst nematode governed by the *Cre* locus have been identified (Williams et al. 1994). This is of special importance for breeding resistant varieties as

the bioassay for nematode resistance screening is expensive and difficult to use in a practical breeding programme.

#### e) Rye and Oats

Both rye and oats are mainly grown in the northern hemisphere and in comparison with wheat and barley they are of minor importance. Concerning rye it has to be stated that in comparison with other cereals the molecular maps are less far developed and that rye is attacked by comparatively few fungal and viral pathogens only. Therefore, breeding for resistance is of lesser importance in this crop and consequently RFLP markers are available for a major gene for powdery mildew resistance on chromosome 1R of rye only (Wricke et al. 1996). However, *Secale cereale* is an important source for resistances to powdery mildew, leaf rust, stem rust and stripe rust. Via translocation many genes like *Pm8*, *Pm 17*, *Sr31*, *Lr26*, *Y49* and many more, as well as resistance to the greenbug and the Hessian fly, have been introgressed into common wheat (cf. Friebe et al. 1996a).

In cultivated oats crown rust caused by *Puccinia coronata* f. sp. *avenae* and stem rust (*Puccinia graminis* f. sp. *avenae*) have to be considered as the most destructive fungal diseases. Using RILs derived from crosses between the diploid species *Avena strigosa* and *A. wiestii* RFLP and RAPD markers for the *Pca* locus of *A. strigosa* conferring race-specific crown rust resistance genes have been developed (Rayapati et al. 1994; Wise et al. 1996). In hexaploid oats (*A. sativa*,  $2n = 6x = 42$ ) tightly linked RAPD markers confirming the feasibility of bulked segregant analysis in polyploid crops have been identified for the crown rust resistance gene *Pc68* (Penner et al. 1993a) and RAPD as well as RFLP markers have been detected for the genes *Pc91* and *Pc92*, respectively (Rooney et al. 1994; Wilson and McMullen 1996). Furthermore, Bush et al. (1994) have identified RFLP markers for three crown rust resistance genes in cultivated oats derived from *A. sterilis*. Recently, markers for *Pc38*, *Pc39* and *Pc48* have been developed by using NILs (O'Donoughue 1996).

Concerning stem rust, RAPD markers for the *Pg3* gene have been identified by Penner et al. (1993b) and RFLP markers for genes *Pg9* and *Pg13* have been detected by O'Donoughue et al. (1996). An overview on the identification, localization and utilization of molecular markers in breeding for rust resistance in oats is given by O'Donoughue (1996).

## 5. Comparative Linkage Mapping and Map-Based Cloning Approaches

Over the past years extensive genetic maps have been developed in major grain crops belonging to different tribes of the family *Graminea* (cf. Sect. 3) and molecular markers for different resistance genes have been identified in each crop, facilitating efficient marker-assisted selection (cf. Sect. 4). Furthermore, comparative mapping experiments have revealed that besides rearrangement, highly conserved blocks showing identical gene order (colinearity) even between species of different tribes are present within the major grain crops, e.g. between rice and barley (Saghai Maroof et al. 1996). An overview focusing on conservation of marker synteny and comparative genetics of major grain crops is given by Moore et al. (1995), Devos et al. (1995) and Wilson et al. (1996).

Besides a more detailed knowledge of genetic aspects involved in the evolution of grasses, comparative linkage maps offer the opportunity of the identification of related genes in different species and the transfer of knowledge from one species to another, thereby providing a large pool of markers perhaps suitable for marker-assisted selection in all grain crops. Consequently, markers for disease resistance identified, e.g. in barley, may be suitable for selection in other *Triticeae* too. This, for example, holds true for leaf rust resistance in cultivated oats and the other *Triticeae* species (Wilson et al. 1996). Furthermore, regions of interest may be saturated by using markers from different species, e.g. rice markers to saturate the *Rpg1* region of barley (Kilian et al. 1995).

Besides this, since large regions of colinearity are present between, e.g., wheat and rice and barley and rice, the latter species may serve as a model plant for the isolation of resistance genes by map-based cloning, because the rice genome (0.88 pg) is about 12 times smaller than the barley (10.1 pg) and about 38 times smaller than the wheat genome (33 pg) and carries a small amount of repetitive DNA only (Arumuganathan and Earle 1991). Therefore, the isolation of resistance genes out of the very complex genomes of wheat and barley may be conducted by chromosome walking or landing in rice. In general, the construction of high density maps around the gene of interest (cf. DeScenzo et al. 1994; Bauer and Graner 1995; Bauer et al. 1996) identifying markers which are at a physical distance from the targeted gene that is less than the average insert size of the genomic library is a paradigm for map-based cloning in plants with large genomes (Tanksley et al. 1995), because chromosome walking strategies are in general hampered by the high amount of repetitive DNA. In this respect, it has to be taken into account that recombination frequencies are not linear along chromosomes and genetic distances in centiMorgans (cM) cannot be directly translated into basepairs (Korzun and Künzel 1996).

Yeast artificial chromosome (YAC) and bacterial artificial chromosome (BAC) libraries have been established in major grain crops (Edwards et al. 1992; Kleine et al. 1993; Umehara et al. 1995; Wang et al. 1995; Woo et al. 1995), but no resistance gene has been isolated out of these complex genomes so far, due to the problems mentioned above. Recently, some resistance genes such as *Pto* (Martin et al. 1993) and *Cf-9* (Jones et al. 1994) in tomato, *N* in tobacco (Whitham et al. 1994) or *RPS2* in *Arabidopsis* (Bent et al. 1994) have been isolated and it was discovered that these genes deriving from different plant species and all including a hypersensitive response to pathogen invasion are sharing similar sequence motifs (for review see Staskawicz et al. 1995). Concerning major grain crops, YACs and BACs linked to the blast resistance genes *Xa-1* and *Xa-21* of rice have been identified recently as being the first step to positional cloning and functional analysis of the respective resistance genes (Wang et al. 1995; Yoshimura et al. 1996).

## 6. Conclusions and Future Prospects

The number of resistance genes tagged by the rapidly developing molecular marker systems has increased dramatically during the past 5 years and will do so in the future. The use of these markers has added a new dimension to breeding for resistance in grain crops, as they facilitate efficient marker-based selection without the need for extensive greenhouse screening tests and field experiments, which in general rely on the maintenance or natural occurrence of the respective pathogen(s).

Furthermore, resistance genes against different pathogens may be easily combined by using these markers or different resistance genes against one pathogen may be assembled in one breeding line (gene pyramiding). This task is very difficult to solve by conventional breeding as many different pathotypes showing different virulences have to be maintained. In the case of the absence of appropriate pathotypes this goal cannot be achieved without the help of molecular markers at all.

Furthermore, molecular markers may help to minimize the linkage drag around DNA segments derived from alien species and may help to calculate the possibilities of combining different resistance genes in one breeding line due to their known map position. However, molecular markers of course will never replace established breeding schemes, but their use within these schemes will help plant breeders to achieve the different goals of breeding for resistance more easily and rapidly.

Besides these more practical aspects of molecular marker systems, comparative linkage mapping and map-based cloning approaches will lead to a more detailed understanding of the molecular basis of disease resistance in grain crops in the near future. Finally, the isolation of individual genes and their transfer by genetic engineering to adapted cul-

tivars will open new ways of a more systematic and directed improvement of crop cultivars.

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## Function of Genetic Material Responsible for Disease Resistance in Plants

By Gerhard Wenzel

### 1. Introduction

Today, the availability of recombinant DNA techniques together with advances in molecular biology and cell culture provides access to a **refined understanding of the genome**. Our present century is moulded by the invention of the genome structure and the subsequent use of this knowledge in genetics and its applied wing: breeding. Starting with the rediscovery of the Mendelian laws, classical segregation analysis and cytology formed the basis of scientific breeding strategies. Complete DNA sequences of many prokaryotes have been determined, the genome of yeast has been sequenced and it is expected that the base pairs of the model plant *Arabidopsis* will be sequenced before the end of this century. Although such a **sequence analysis provides the most complete information** about the genetic basis, it does not inform about the **meaning and the functionality of genes**. Taking into consideration the enormous size of the genome and the fact that a tremendous number of the base pairs are silent, it seems recommendable to analyse only those parts containing information.

To find such areas several approaches have been elaborated, making use of phenotypic segregations and correlations of such phenotypes to molecular linkage maps. A compromise is the analysis of DNA fragments generated by restriction enzymes gearing the development of this research area. Progress in analysing the higher plants' genomes is driven by two goals: (1) finding DNA probes closer and closer linked to a phenotype, and (2) making these selection tools so easy that they can be used under applied aspects.

The increasing amount of information documented in **dense gene maps** together with an excellent **bioinformation system** allows increasingly calculations about the function of genes (Michelmore 1995; Jones 1996). Particularly under the aspect of **synteny**, comparisons will be possible, probably elucidating common principles, e.g. in defence mechanisms against pests, or in the development of morphological structures. In the area of secondary product formation already a wide range of information about the biochemical pathways exists (Henry et al. 1996) and increasingly the corresponding genes are grouped to the responsible en-

zymes. Two areas are still unclear: (1) the molecular architecture influencing plant yield, the morphology or the function of sexual organs, and (2) the function of genes responsible for resistances. Since the first topic is covered by Thießen and Saedler (this Vol.), this chapter will focus on the advances in mapping and understanding the functionality of genes responsible for disease resistance.

Breeders have used monogenic disease resistance R-genes in their efforts to produce resistant varieties. The R-genes enable plants to recognize specific races of pathogens and to react with a specific defence response. However, races of pathogens with new virulences evolve that can overcome individual R-genes. Since now the structure of increasing numbers of such R-genes is elucidated, progress related to a functional understanding of the host pathogen interaction is anticipated. Here, the development will be discussed under the aspects of how and where a contribution of the research to the function of genes responsible for resistance is growing.

## 2. The Technique

Diversity at the phenotypic level is caused by corresponding differences in the DNA sequence. The availability of recombinant DNA techniques provides access to a refined analysis of the genome. Point mutations, insertions, deletions or inversions cause differences in the nucleotide sequence and variability in the length of individual restriction fragments. The altered fragment lengths can be detected by gel electrophoresis – or most recently also by optical means (Anantharaman et al. 1977) – and result in restriction fragment length polymorphisms (RFLPs). The procedures used for the genome and gene identification include chromosome walking, megabase techniques, as well as tagging and c-DNA approaches.

RFLP probes may be converted into sequence tagged sites (STS; Blake et al. 1996) or specifically cleaved amplified regions (SARS; Paran and Michelmore 1993). This allows direct visualization of the DNA in the gels without the need of labeling; an important advantage in applied work.

The RFLP method advanced to the very powerful amplified fragment length polymorphism (AFLP) technique (Vos et al. 1995), allowing much denser maps by identifying very small differences in the genome. Another approach, the randomly amplified polymorphic DNA sequences (RAPD) (Williams et al. 1991), is based on genomic DNA fragments bordered by defined primers which are amplified during a polymerase chain reaction (PCR).

The RAPD technique can be performed with any conceivable primer sequence comprising about ten nucleotides and containing approximately 50% cytosine and guanine residues. While the advantage of this technique is that polymorphisms can be detected directly upon size fractionation on a gel without expensive and time-consuming hybridi-

zation procedures, the reliability in different genetic backgrounds is only limited. For RAPDs the problem appears that dominant markers show repulsion linkage to the resistance gene. In those cases the RAPD marker has to be transferred into sequence characterized amplified regions (SCARs), e.g. in pea for the *Erisiphe pisi* resistance (Dirlewanger et al. 1994). An exception is the RAPD marker for *Verticillium* in tomato: it is codominantly inherited and directly differentiates between the resistant and susceptible allele (Kawchuck et al. 1994).

Additionally, **microsatellites**, small conserved base sequence patterns distributed rather evenly over the genome, can be incorporated in those instances where no other polymorphisms are detected (Hearne et al. 1992). While RFLP, RAPD and AFLP marker systems depend predominantly on anonymous DNA sequences, the microsatellite technique uses defined sequence motives of two or four base pairs. In eukaryotic genomes microsatellites express a highly dispersed distribution. By PCR the different sizes of microsatellite loci can be easily detected. Due to their high amount of information, they are a useful marker system, especially for species with low genetic diversity, e.g. wheat (Röderer et al. 1995). These new marker techniques, particularly the microsatellites and AFLPs, have not yet been used for marker-aided selection, but for producing dense maps aiming at the identification of genes by chromosome landing (Tanksley et al. 1995).

Since in gene identification the application of molecular markers demands the need to know the localization of the marker in the genome, **genetic linkage maps** are an additional prerequisite for the localization and the analysis of gene functions. Often for a securer gene identification, the bulked segregant analysis (BSA), doubled haploids (DHs), nearly isogenic lines (NILs) or recombinant inbred lines (RILs) are used as mapping populations.

### 3. Presently Mapped Major Resistance Genes

For the world's most important plant pathogens, the fungi, up till now progress in applying molecular procedures has been rather slow. The understanding how the **host/pathogen** interaction works is a prerequisite in order to start unconventional breeding programmes. The presently possible **transformation** of genes for the expression of antifungal proteins or for an overexpression of phytoalexins is normally not sufficient to protect the plant under field conditions (Hain et al. 1993). Thus, additional knowledge is needed.

The techniques to identify and use viral genes, e.g. the viral coat protein gene, the anti-sense RNA for virus resistance, the movement proteins and the replicase mediated resistance, as well as the strategies against bacterial pathogens are already summarized by Horn et al. (1996) in a previous volume.

Table 1. Summary of dicot host plants and diseases for which DNA probes are available, together with a reference where further information can be found

Host	Disease	Number of alleles identified	Technique	Reference
<i>Arabidopsis</i>	<i>Pseudomonas syringae</i>	2	Tagging, RFLP	Simonich and Innes (1995)
	<i>Peronospora parasitica</i>	5	RAPD, STS	Tör et al. (1994)
Beans	<i>Colletrochum lindemuthianum</i>	1	NIL, RFLP, SCAR, RAPD	Adam-Blondon et al. (1994)
	Common bean mosaic virus	1	NIL, RAPD	Haley et al. (1994)
	<i>Uromyces appendiculatus</i>	3	NIL, RAPD	Johnson et al. (1995)
Cucumber	<i>Cladosporium cucumerinum</i>	1	RFLP	Kennard et al. (1994)
	<i>Pseudoperonospora cubensis</i>	1	RFLP	Kennard et al. (1994)
Flax	<i>Cochliobolus carbonum</i>	3	Tagging	Lawrence et al. (1995)
Lettuce	<i>Bremia lactuca</i>	19	BSA, STS, RAPD, RFLP	Kesseli et al. (1994)
	<i>Plasmopara lactucae</i>	1	RAPD	Robbins et al. (1994)
	Turnip mosaic virus	1	BSA, RAPD	Robbins et al. (1994)
Pea	<i>Erysiphe pisi</i>	2	RIL, RFLP, RAPD, SCAR	Dirlwanger et al. (1994)
	<i>Fusarium oxysproum</i>	1	RFLP, Microsatellite	Dirlwanger et al. (1994)
	Pea mosaic virus	1	RFLP, Microsatellite	Dirlwanger et al. (1994)
	Seed-borne mosaic virus	1	RFLP, RAPD	Timmerman et al. (1993)
Potato	<i>Globodera rostochiensis</i>	3	NIL, RFLP, RAPD, AFLP	Ballvora et al. (1995)
	<i>Phytophthora infestans</i>	2	DH, BSA, RFLP, AFLP, QTL, RFLP	El-Karbotly et al. (1996)
	Potato virus X	2	RFLP	Leonardis-Schippers et al. (1994)
	Potato virus Y	1	RFLP	Ritter et al. (1991)
				Hämäläinen et al. (1997)



Table 1 (continued)

Host	Disease	Number of alleles identified	Technique	Reference
Rape seed	<i>Plasmodiophora brassica</i>	1	RFLP, AFLP	Voorrips et al. (1997)
Soybean	<i>Heterodera glycines</i>	1	RIL, RFLP	Webb et al. (1995)
	<i>Phytophthora megasperma</i>	3	NIL, RFLP	Diers et al. (1992)
	Soybean mosaic virus	1	Microsatellite, RFLP	Y.G. Yu et al. (1996)
Sugar beet	Rizomania	1	RFLP	Barzen et al. (1995)
	<i>Heterodera schachtii</i>	3	RFLP, RAPD	Salentijn et al. (1995)
	Beet necrotic yellow vein virus	1	RAPD	Scholten et al. (1997)
	<i>Cladosporium fulvum</i>	6	NIL, BSA, RFLP, AFLP	Thomas et al. (1995)
Tomato	<i>Fusarium oxysporum</i>	3	NIL, RFLP	Sarfatti et al. (1991)
	<i>Leveillula taurica</i>	1	RFLP	Chungwongse et al. (1994)
	<i>Macrosiphum euphorbiae</i>	1	RFLP, RAPD, STS	Kaloshian et al. (1995)
	<i>Oidium lycopersicon</i>	2	NIL, RFLP, RAPD	Yaghoobi et al. (1995)
	<i>Verticillium dahliae</i>	1	BSA, RFLP, RAPD	van der Beek et al. (1994)
	Potato virus X	1	NIL, RAPD	Kawchuk et al. (1994)
	Potato spotted wilt virus	1	RFLP	Ritter et al. (1991)
	Tomato mosaic virus	1	NIL, RFLP, RAPD	Stevens et al. (1995)
	Tomato yellow leaf curl virus	1	RFLP	Tanskley et al. (1992)
	Tomato yellow leaf curl virus	1	RFLP	Zamir et al. (1994)

Table 2. Summary of monocot host plants and diseases for which DNA probes are available, together with a reference where further information can be found

Host	Disease	Number of alleles identified	Technique	Reference
Barley	<i>Erysiphe graminis</i>	10	NIL, RFLP, DH, AFLP	Schönfeld et al. (1996), Büschges et al. (1997), Backes et al. (1997)
	<i>Puccinia graminis</i>	QTL	DH, BSA, RFLP	Kilian et al. (1995)
	<i>Puccinia hordei</i>	2	BSA, RAPD	Poulsen et al. (1995)
	<i>Puccinia striiformis</i>	1	RFLP	Chen et al. (1994)
	<i>Rhynchosporium secalis</i>	QTL	DH, NIL, RFLP	Graner and Tekauz (1996)
		4	DH, RFLP	Backes et al. (1995)
	<i>Pyrenophora teres</i>	QTL	DH, RFLP	Graner et al. (1997b)
		1	DH, RFLP	Steffenson et al. (1996)
	<i>Cochliobolus sativus</i>	QTL	RFLP	Steffenson et al. (1996)
		1, QTL		
Maize	<i>Typhula incarnata</i>	1	RFLP	Graner et al. (1997a)
	<i>Pyrenophora graminea</i>	QTL	RFLP	Pecchioni et al. (1996)
	Barley yellow dwarf virus	1	RFLP	Collins et al. (1996)
	Barley stripe mosaic virus	1	DH, RFLP	Edwards and Steffenson (1996)
	Barley yellow mosaic virus	7	DH, RFLP, RAPD	Graner (1996)
	<i>Heterodera avenae</i>	1	RFLP	Langridge (cit. In Graner 1996)
	Maize dwarf mosaic virus	2	RFLP	Ming et al. (1997)
	<i>Bipolaris maydis</i>	1	RFLP	Zaitlin et al. (1993)
	<i>Puccinia sorghi</i>	1	NIL, RFLP	Hulbert and Benetzen (1991)
	<i>Cercospora zeae-maydis</i>	QTL	RFLP	Bubeck et al. (1993)
	<i>Colletotrichum graminicola</i>	QTL	RFLP	Jung et al. (1994)
	<i>Helminthosporium truncicum</i>	1	NIL, RFLP	Bentolia et al. (1991)

Table 2 (continued)

Host	Disease	Number of alleles identified	Technique	Reference
Oats	<i>Puccinia coronata</i>	1	BSA, RAPD	Penner et al. (1993a)
	<i>Puccinia graminis</i>	1	NIL, RAPD	Penner et al. (1993b)
Rice	<i>Orseolia oryzae</i>	1	RIL, BSA, RFLP, RAPD	Mohan et al. (1994)
	<i>Pyricularia oryzae</i>	3	NIL, RFLP	Miyamoto et al. (1996)
	<i>Pyricularia grisea</i>	2	NIL, RFLP	Z.H. Yu et al. (1996)
	Rice tungro virus	1	RFLP, RAPD	Sebastian et al. (1996)
	<i>Xanthomonas oryzae</i>	6	NIL	Williams et al. (1996)
Sorghum	<i>Sporisorium reilianum</i>	1	RFLP, RAPD	Oh et al. (1994)
Wheat	<i>Heterodera avenae</i>	2	BSA, NIL, RFLP, RAPD	Eastwood et al. (1994)
	<i>Mayetiola destructor</i>	11	RFLP	Dweikat et al. (1997)
	<i>Puccinia recondita</i>	3	RFLP, RAPD, STS	Feuillet et al. (1995)
	<i>Puccinia graminis</i>	1	RFLP	Paull et al. (1994)
	<i>Erysiphe graminis</i>	8	NIL, RFLP	Hardt et al. (1995)
	Wheat streak mosaic virus	1	STS, RAPD	Talbert et al. (1996)

As a first central step in the direction of identifying resistance genes, during recent years a rapidly increasing number of monogenic, race-specific genes showing **gene-for-gene interaction** have been mapped in economically important dicot (Table 1) and monocot (Table 2) species. This demands the production of mapping populations and the skills for exact phenotypic evaluations.

For host solidus pathogen interactions with fungi imperfecti, e.g. for beans and *Colletotrichum lindemuthianum*, the proof of a gene-for-gene interaction is missing of course. Due to the formation of races and the independent inheritance of seven dominant genes for resistance against this fungus in beans, Adam-Blondon et al. (1994) grouped a gene (*Are*) to the category of race-specific genes. They even found closely linked markers in NILs, which now can be used for marker-aided selection. Sometimes the genes identified are not resistance genes against the pathogen itself, but for a vector, e.g. the gene for rice tungro virus resistance which does not act against the virus but rather causes insect resistance against *Tetigonia viridissima*, the responsible virus vector (Sebastian et al. 1996).

Besides the race-specific genes, an increasing number of **quantitatively inherited genes**, quantitative trait loci, (QTLs) are localized (Dirlewanger et al. 1994; Backes et al. 1995, 1997). The identification of polygenes for disease resistance is not different from the identification of other polygenic traits. For review of the basic principles see Tanksley et al. (1995). Some characterizations of polygenic traits are incorporated in Table 1 and 2. To find correlations between geno- and phenotype the progeny is divided into several subpopulations depending on the allele groups of a trait (e.g. parental type, heterozygotes). A linkage between a QTL and a genetic marker is given when the phenotypic means of a class of markers are significantly different. The most commonly used procedure for mapping QTLs is today interval mapping according to Lander and Botstein (1989), where chromosome segments flanked by two markers are analysed. For the identification of gene functions, presently only the race-specific genes are under investigation.

#### 4. Genomic Organization of Resistance Genes

Although the knowledge about the number and genetic localization of disease resistance genes is still incomplete, the knowledge on the genomic organization of the first genes is rapidly growing. Evidently the **resistance genes are not evenly distributed along the chromosomes** but rather tend to **form clusters**. These are either composed of different specificity or of genes that condition resistance against one pathogen. The presence of heterospecific clusters has been described for tomato, wheat (Ellis et al. 1995) and barley (Graner et al. 1996). Homospecific clusters are more common (Mahadevappa et al. 1994). In barley particularly the *Mla* locus represents an extreme example of multiple allelism

(Jahoor et al. 1993). Examples of other complex resistance genes have been studied extensively in flax where the *L* locus confers resistance to rust exhibiting multiple allelism.

The physical analysis of the gene sequence reveals that its 3' region consists of a stretch of tandem repeated motives, the repeat number of which differs in the alleles analysed (Ellis et al. 1995). It seems that variability in repeat number results in the generation of a new allele with altered specificity. Thus, one may speculate that the genetic variability of the *Mla* locus in barley is accounted for by a similar mechanism. Differences may be also a result of gene amplification of an ancestral gene by unequal crossover events mediated by flanking repetitive elements (Ellis et al. 1995). Such questions may be solved by isolating the gene and subsequently identifying its functions.

## 5. Gene Isolation

After gene mapping, for gene identification, marker-based chromosome walking techniques are applied predominantly. Additionally, **tagging techniques using increasingly transposon induced mutant populations** (Osborne and Baker 1995) together with cDNA approaches are gaining importance under this aspect. For the walking technique high resolution maps have been constructed, allowing the saturation of the relevant chromosomal region with very closely linked markers. The closest ones will be used to select homologous clones from large insert libraries which in turn allow the construction of physical maps around the genes. An alternative strategy for the isolation of disease resistance genes exploits the observation that many resistance genes isolated in one plant species **share similar sequences or represent members of comprehensive and widespread gene families**.

Thus, isolation and mapping of homologous clones may lead to identification of candidates, which have to be further tested by genetic analysis. Particular examples for this approach are heterologous probes from plants like *Arabidopsis* or rice with small genomes but a huge amount of information available.

For resistance genes, common features like genes for **enzymes rich in leucine** (leucine-rich repeats, LRRs) or enzymes responsible for signal transductions are of a very great help. The information available – though still very limited – allows first speculations on the type of function of the genes identified.

## 6. Genes Presently Cloned

Eighteen genes responsible for disease resistance have been cloned up till now (Table 3). All are following the gene-for-gene hypothesis, and

Table 3. Common structural characteristics of proteins of cloned genes for resistance

Group	Protein	Host/pathogen	Structure	Reference
I	PTO	Tomato/ <i>Pseudomonas</i>	Intracellular serine/threonine kinase membrane bound	Martin et al. (1993)
	PT11	Tomato/ <i>Pseudomonas</i>	Serine/threonine kinase phosphorylated by PTO, interacting with PTO	Zhou et al. (1995)
IIa	RPS2	<i>Arabidopsis/Pseudomonas syringae</i>	Intracellular protein with leucine zipper, nucleotide binding site, leucine-rich-repeats	Bent et al. (1996)
	RPM1 PRF	<i>Arabidopsis/Pseudomonas syringae</i>		Mindrinos et al. (1994) Grant et al. (1995)
IIb	N	Tobacco/TMV	Intracellular protein	Salmeron et al. (1996)
	L2, L6, L10 RPP5 RPP14	Flax/ <i>Cochliobolus carbonum</i> <i>Arabidopsis/Peronospora parasitica</i>	1L-1R homology, nucleotide binding site leucine-rich repeats	Whitman et al. (1994)
III	Cf-2 Cf-4 Cf-5 Cf-9 I2	Tomato/ <i>Cladosporium fulvum</i> Tomato/ <i>Fusarium oxysporum</i>	Transmembrane proteins with extracellular leucine rich repeats	Lawrence et al. (1995) Parker et al. (1996) Jones et al. (1996)
IV	Xa21	Rice/ <i>Xanthomonas oryzae</i>	Transmembrane protein with intracellular kinase and extracellular leucine-rich repeat	Jones et al. (1996) Jones et al. (1996) Jones et al. (1996) Song et al. (1995)
V	Mlo	Barley/ <i>Erysiphe graminis</i>	Transmembrane proteins nuclear localized	Büschges et al. (1997)

can be grouped into four sections according to the possible function of the proteins resulting from their DNA sequence. Most of these proteins are incorporated within the **ligation and/or in the signal transduction** (De Wit 1995). Fifteen encode an LRR motif. **This motif could not only explain recognition specificities but also allow their rapid evolution.** R-gene products might be explained to have two functions: molecular recognition and activation of plant defence upon recognition. The products may fall again into two classes, recognizing either **extra- or intracellular pathogen derived ligands**. It is, however, not yet clear whether the R-gene products interact directly with the avirulence gene (*Avr*-gene)-coded elicitors, or whether the subcellular localization can be deduced for their primary sequence. Furthermore, type and number of additional plant genes which are necessary for the signal transduction of the R-gene are unknown. **It is striking that R-genes for a wide range of pathogens of different plant species code for structurally similar proteins.** This similarity makes probable a high amount of mechanical conservation of the signal transduction chains used for the induction of reaction against pathogens (Bent 1996).

#### a) Intracellular Protein Kinase (Group I)

A common feature of proteins of group I is the **membrane bound serine solidus threonine kinase**. The first described and dominating example is the gene *Pto* of tomato causing resistance against the bacterium *Pseudomonas*. It codes a functional serine solidus threonine kinase (Loh and Martin 1995a,b). Subsequently, a second gene of the *Pto*-gene family, linked in a 400-kb region, was isolated causing sensitivity against the insecticide fenthion. It is also a serine/threonine kinase (Rommens et al. 1995). This fenthion sensitivity (*Fen*) gene expresses at the protein level 80% identity to *Pto*. Neither possess a region pointing to an extracellular or transmembrane localization but a possible site for membrane association in a number of proteins including protein kinase (Grand 1989). Consequently, both proteins can act with a postulated membrane bound receptor (Loh and Martin 1995a), and express homology to different serin/threonin kinases, including the transmembrane protein S-receptor kinase (SRK6) from *Brassica*. This probably codes for a receptor kinase coupled together with the S-locus-glycoprotein (SLG), a glycoprotein of the cell wall coded by the incompatibility locus S.

The S-incompatibility is located in the papillar cells of the stigma which detects probably specific structures of the pollen surface causing self-incompatibility in *Brassica*. This incompatibility is based on a gene-for-gene reaction like the *Pto/avrPto* interaction (Dickinson 1996).

Zhou et al. (1995) isolated using the yeast two hybrid system (Fields and Song 1989) the additional serine/threonine kinase, PTO-interacting (PTI1) which is phosphorylated by PTO. The authors proposed a hypothetical signal transduction chain, in which at first an elicitor produced by an avirulent bacterium interacts directly or indirectly with PTO and then phosphorylates via PTI transcription factors, which activate disease-relevant genes. By the resulting production of a protein the hypersensitive reaction is started.

Since *Pti* belongs to a gene family a protein homologous to PTI might exist, which is activated by FEN using the same transduction pathway. In the meantime, further PTO-interacting proteins were isolated showing homologies to transcription factors of tobacco (Bent 1996).

These proteins are similar to DNA binding proteins, identifying a conserved sequence at the promoters of pathogen-related (PR) protein genes. A PR box binding has been verified experimentally, which hints at a mechanism for expressing disease-relevant proteins coupling by this the detection of an avirulent pathogen with the expression of resistance genes.

#### b) Intracellular Proteins with a Nucleotide Binding Site (NBS) and C-Terminal LRRs (Group II)

The next three groups have the character LRR in common. LRRs might provide a general mechanism for providing both a regular protein structure on which to elaborate recognitional specificity and a DNA structure that because of these LRRs might have the capacity to rapidly evolve new specificities by unequal crossing over (Jones et al. 1996). Furthermore, LRRs are common proteins that interact with other proteins. It is not unreasonable to consider their role as analogous to that of the antibody variable domain. However, in plant selection for useful recognitional specificities this is only exercised germinally, unlike somatic selection exercised on the diversity generated in the mammalian system.

An LRR defines a turnable protein binding domain with 24 to 26 amino acids (Kobe and Deisenhofer 1994). They often appear in repeats of 1 to 40 and in functional and evolutionary different protein families, all of which are part of a protein-protein interaction and normally also part of signal transduction pathways, e.g. the transmembrane kinase (TMK1) or the receptor-like kinase (RLK5) in *Arabidopsis* (Walker 1993) or an LRR protein of unknown function detected in tomato with increased concentrations in infected plants (Toreno et al. 1996).

It is assumed that the LRR domain is the recognition and binding site perhaps of Avr proteins. The specificity of the LRRs depends probably



less on the conserved repeatedly appearing hydrophobic groups present in the inner protein and responsible for tertiary structure but rather from the interspersed exposed variable amino acids (Bent 1996). The organization of repeated sequences and the appearance of defect mutants as a consequence of intragene rearrangements or point mutations in R-genes in these domains (Grant et al. 1995; Parker et al. 1996) are hints at how on the molecular level new configurations and in consequence new specificity may appear.

The presence of a nucleotide binding site makes it probable that the resistance genes of group II need ATP or GTP for their function (Traut 1994). This hypothesis is backed by preliminary results of directed mutagenesis of the NBS-consensus sequence which eliminates the hypersensitive reaction (HR) induced by RPS2 and N (Bent 1996). The function of the NBS domain is still unknown. It is possible, however, that an alteration of the interaction of R-proteins with other members of the signal transduction chain is responsible.

The resistance genes *Rps2*, *Rpm1* and *Rpp5* from *Arabidopsis*, the N-gene from tobacco mosaic virus (TMV)-resistant tobacco, *L6* from flax and *Prf* from tomato against different bacterial, viral and fungal pathogens; despite these differences, they have in common to code for cytoplasmic proteins which contain LRRs, and an NBS often called a P-loop. Within this group of R-genes the genes *Rps2*, *Rpm1* and *Prf* form a subgroup since they have in common a heptameric repetitive sequence motive, the so-called leucine zipper between N-terminus and the NBS and LRR domain.

This consensus sequence improves the protein-protein interaction and allows the homo- and heterodimerization of eukaryotic proteins (Alber 1992). It is not understood which role it has for the function of the R-genes, but experiments using the yeast-two-hybrid system (Fields and Song 1989) for the search of interacting compounds for the R-proteins are in progress (Bent 1996). The *Rpm1*-gene is active against two independent avirulence genes, *avrRpm1* and *avrB* of *Pseudomonas syringae* pv. *maculicola* or pv. *glycinea*. If *Rpm1* codes for a receptor, it should be probable that only one or overlapping binding sites are responsible for both *avr*-gene products, since no mutants of *Arabidopsis* were found separating both specificities. Alternatively, a double specificity by the interaction of RPM1 with a general *avr*-receptor might be the reason (Grant et al. 1995). For the gene products of the two avirulence genes *avrRpm1* and *avrRpt2* from *Pseudomonas syringae*, a common factor is proposed for which both compete. This would also explain the epistasy of the two *avr*-genes (Reuber and Ausubel 1996; Ritter and Dangl 1996).

Since RPS2 is located in the cytoplasm demonstrated by mutation of a responsible membrane domain and RPM1 is not transmembrane, the responsible factor might be a membrane protein acting between the extracellular *avr*-gene products and the intracellular R-gene products (Innes 1996). A possible candidate is the bacterial protein harpin, coded by the *hrpZ* gene secreted from the bacterium. When it is injected to tobacco, necrosis is induced (He et al. 1993; Huang et al. 1995). However,

also the interaction of the bacterial avr-B-protein with the corresponding product of the resistant plant within the plant cell explains why at least some products of the resistance gene are located in the cytoplasm (Gopalan et al. 1996).

To group IIb belong the proteins N, L2, L6, L10, RPP5 and RPP14.

They have homologies to the cytoplasmic domains of the interleukin-1-receptor (IL-1) of mammals and the Toll-transmembrane protein from *Drosophila*, both inducing a signal transduction path resulting in the activation of immune genes in mammals and the function of the dorso-ventral polarity in the embryo of *Drosophila* (Dangl 1995). Recent studies demonstrated that a Toll/IL-1R-signal transduction path improves the immune response of adult flies after pathogen attack. It results in the transcriptional activation of genes for antimicrobial peptides (Lemaite et al. 1996).

It is possible that the conserved N-terminal part of the resistance genes *N*, *L6* and *Rpp5* is an effector domain, inducing a Toll-similar signal cascade (Parker et al. 1996). From *N*, *L6* and *Rpp5* reduced transcripts can be produced by alternative splicing or by the expression of a variant gene which consists only of the Toll homologous N-terminal part and the NBS. As proposed for truncated *N*, such transcripts might function as dominant regulators of resistance proteins via the stochastic binding of nucleotides (Dinesh-Kumar et al. 1995). The anticipated intracellular localization of *N* is understandable, since the life cycle of TMV happens in the cytoplasm of the host cell. In contrast to *N*, the proteins *L2*, *L6* and *L10* might be signal peptides secreted by the responsible gene product (De Wit 1995; Lawrence et al. 1995).

The *Rpp5* and *Rpp14* genes have high N-terminal homology to the tobacco *N* gene and the *L* genes of flax. This finding supports the hypothesis that different specificities have arisen from common ancestral genes (Staskawicz et al. 1995). Also, the similarities between certain functional motives suggest that resistance genes recognizing different pathogens with strikingly different modes of attack operate through similar, if not identical, pathways. Parker et al. (1996) isolated several mutations at the *Rpp* loci and could identify with this approach the correct open reading frame.

#### c) Extracytoplasmic Proteins with a Transmembrane Domain (Group III)

The four tomato genes *Cf-2*, *Cf-4*, *Cf-5* and *Cf-9* active against different races of the fungus *Cladosporium fulvum* form a third group to which also the resistance gene *I2* from tomato active against *Fusarium oxysporum* belongs (De Wit 1995). They are probably transmembrane proteins containing a small cytoplasmic and a larger extracytoplasmic, glycosylated domain, and consist primarily of 28 extracytoplasmic LRRs

attached at the C-terminus to the cell by a transmembrane domain and a short cytoplasmic domain. The C-terminal half of the LRR domain of several *Cf* genes shows substantial homology. This conserved domain might interact with the extracytoplasmic domain of another protein to effect signal transduction. Differences between *Cf* genes are mostly confirmed to the N-terminal half of the LRRs, suggesting that this domain may play a role in the specific recognition (Jones et al. 1996). Comparisons of the sequence of *Cf-2* and *Cf-9* demonstrated homologies at the C-terminal end containing the transmembrane domain and a part of the extracellular LRR domain responsible for the ligation.

Dixon et al. (1996) proposed two possible models for these two R-genes:

1. The avr-protein might bind at the LRR domain and this complex might react with a membrane-bound NADPH-oxidase which starts the resistance reaction of the plant by producing superoid anions.
2. A complex of R- and avr-proteins might bind a transmembrane kinase which activates in additional reactions that NADPH-oxidase.

Several examples for receptor protein kinases bound in membranes exist in plants, e.g. the RLK5 in *Arabidopsis* (Walker 1993) or the product of the resistance gene *Xa21* of rice (Song et al. 1995).

#### d) Extracytoplasmic LRRs with a Transmembrane Protein Kinase (Group IV)

In Group IV, the resistance gene *Xa21* causing resistance against the bacterium *Xanthomonas oryzae* combines characteristics of groups I and III and contains an intracellular protein kinase as well as extracellular LRRs, linked via a transmembrane domain. The extracellular part of *Xa21* has homologies to *Cf-9* and *Cf-2*, while the kinase domain is similar to PTO. These similarities made it probable that for each resistance gene containing a kinase domain, additionally an LRR-protein similar to *Cf-9* (protein) is necessary, while for other LRRs containing resistance genes, e.g. the genes of group II, a protein kinase is available (Bent 1996). Along this line for the protein kinase PTO an LRR-protein (PRF) was found that interacts with PTO and is necessary for the expression of the resistance (Salmeron et al. 1996).

#### e) The Mlo Powdery Mildew Resistance Gene of Barley (Group V)

A fifth type of the function in inducing resistance is coupled to the powdery mildew resistance gene *mlo* of barley. Mutation-induced recessive alleles (*mlo*) of the barley *Mlo* locus confer a leaf lesion phenotype and

**broad spectrum resistance to *Erysiphe graminis*** (Büschges et al. 1997). Analysis of mutagene-induced *mlo* alleles revealed mutations leading to alterations of the deduced *Mlo* wild type protein. Susceptible intragenic recombinants isolated from *mlo* heteroallelic crosses show restored *Mlo* wild-type sequences. **The deduced amino acid sequence reveals no homologies to any other described plant resistance gene.** However, significant homologous sequences have been found to rice and *Arabidopsis* (Büschges et al. 1997). This strongly suggests that the *Mlo* protein is likely to represent a member of a separate protein family and implies a conserved function among plants.

The large protein segment between predicted transmembrane helices is likely to face the cytosol whereas the C-terminal end appears to be located on the extracellular face (Hartman et al. 1989). In addition, a putative nuclear localization sequence motive (NLS) was found, indicating a possible transport of the protein into the nucleus (Nigg et al. 1991). It is not yet clear whether the protein is located in the nuclear membrane. An anticipated frame shift is predicted to shorten the length of the expressed *Mlo* protein by 75%.

Büschges et al. (1997) assume that this resistance allele represents a complete functional inactivation of the protein whereas the alleles might encode proteins with residual activity. The results show that resistance to *Erysiphe* is caused by a defective *Mlo* gene. For the explanation of the protein action two alternatives exist: (1) *Mlo* has a negative control function in leaf cell death. It would suppress a default cell suicide programme in foliar tissue; and (2) *Mlo* has a specific negative regulatory function by down-regulating multiple defence-related function.

**Spontaneous cell death in *mlo* genotypes represents the end of an accumulating activation of defence responses.** It is concluded that a complete or partial inactivation of the *Mlo* protein primes or upregulates the responsiveness of the seedling for the onset of pathogen defence (Büschges et al. 1997).

#### f) Additional Genes

Most resistance genes cloned up till now take part in ligand binding and/or the signal transduction via phosphorylation cascades. To understand the different pathways leading from the identification of the *Avr*-gene product finally to the resistance reaction of the plant, the different components of this system and their interaction have to be elucidated. Up till now only very few additional genes the product of which acts directly or indirectly with the R-gene products have been found. This may be due to two reasons: (1) The signal transduction chains, resulting in resistance, contain only few components; and (2) several proteins

Table 4. Loci required for disease resistance (Altered from Hammond-Kosack and Jones 1996)

Plant	Locus	R-gene	Pathogen	Loss of function	Localization	Reference
Tomato	<i>Prf</i>	<i>Pto/Fen</i>	<i>Pseudomonas syringae</i>	Complete	Linked with <i>Pto/Fen</i>	Salmeron et al. (1996)
	<i>Rcr-1, Rcr-2</i>	<i>Cf-9</i>	<i>Cladosporium fulvum</i>	Partial	Unlinked	Hammond-Kosack et al. (1994)
	<i>Rcr-3, Rcr-5</i>	<i>Cf-2</i>		Partial – Complete	Unlinked	Hammond-Kosack and Jones (1996)
Arabidopsis	<i>Ndr 1</i>	<i>Rps2</i>	<i>P. syringae</i> pv. <i>toamto</i>	Complete	Unlinked	Century et al. (1995)
		<i>Rpm1</i>	<i>P. syringae</i> pv. <i>glycinea</i>			Century et al. (1995)
		<i>Rpps</i>	<i>Peronospora parasitica</i>			Century et al. (1995)
	<i>nim1</i>	<i>Rpps</i>	<i>Peronospora parasitica</i>	Complete	Unlinked	Delaney et al. (1995)
	<i>Eds1</i>	<i>Rpps</i>	<i>Peronospora parasitica</i>	Complete	Unlinked	Parker et al. (1996)
Barley	<i>Rar 1, Rar 2</i>	<i>Mla-12</i>	<i>Erysiphe graminis</i>	Nearly complete	2H, unlinked	Freialdenhoven et al. (1996)
	<i>Ror 1, Ror 2</i>	<i>mlo</i>	<i>Erysiphe graminis</i>	Nearly complete	Unlinked	Büschges et al. (1997)

might be involved, which are not yet found by tagging or their absence is lethal.

Table 4 summarizes genes identified by mutagenesis which are necessary for the function of specific R-genes. In tomato, several genes required for *Cladosporium resistance* (*Rcr*) were identified in mutagenized homozygous *Cf-9* or *Cf-2* plants. In these mutants, the *Cf* gene function is partially or completely inhibited but there is no linkage (Hammond-Kosack et al. 1994). Another locus, *Ndr1*, the non-race-specific disease resistance against *Pseudomonas*, was detected on chromosome 3 of *Arabidopsis*. **This makes it possible that reactions against fungi and bacteria may rely on identical genes** (Century et al. 1995).

Additional loci required for disease resistance of *Arabidopsis* are *nim1* and *Eds1*, influencing resistance against *Peronospora parasitica* (Delaney et al. 1995; Parker et al. 1996). In barley, loci were identified necessary for the function of powdery mildew resistances. Mutation in *Rar1* and *Rar2* required for *Mla* resistance originally named *Nar1* and *Nar2* reduce the HR production and the induction of disease-relevant gene *Mlat-12*, but not in combination with *Mlg* (Freialdenhoven et al. 1994). The two other loci *Ror1* and *Ror2*, required for *mlo* resistance, inhibit the horizontal *mlo* resistance and the production of papillae (Freialdenhoven et al. 1996). This makes it probable that in barley the resistance against different powdery mildew isolates, based on specific resistance genes, relies on different mechanisms.

Cell death caused by the HR has several similarities with the programmed cell death, apoptosis, observed in mammals and insects (Greenberg et al. 1994). Mutants for cell death have been identified in maize, rice, tomato, barley and *Arabidopsis*. Probably the wild-type genes code for several components of the signal transduction chain resulting in a normal resistance (Jones and Dangl 1996). It is, however, also possible that the mutants cause **unspecific disturbances of the biochemical pathway** (Dietrich et al. 1994).

## 7. Use of Knowledge Deduced from Genome Analysis in Breeding

The fact that most durable resistances are not coded by a single gene but rather by oligo- or polygenic ones is a reason that under applied aspects marker assisted selection (MAS) is **opening up faster success than the transfer of isolated or even monogenic genes, the function of which is known**. The analysis of QTLs is under rapid development. The whole character will often depend on some major alleles, some of which can be identified. The use of selectable markers will allow a more efficient manipulation of resistance genes during the breeding process. Thus, traits with low heritability can be selected more efficiently. **Regarding the realization of gene pyramiding concepts, MAS can replace extensive virulence tests** (Graner et al. 1995). The use of molecular markers will facilitate the combination of resistance genes which due to the lack of appro-

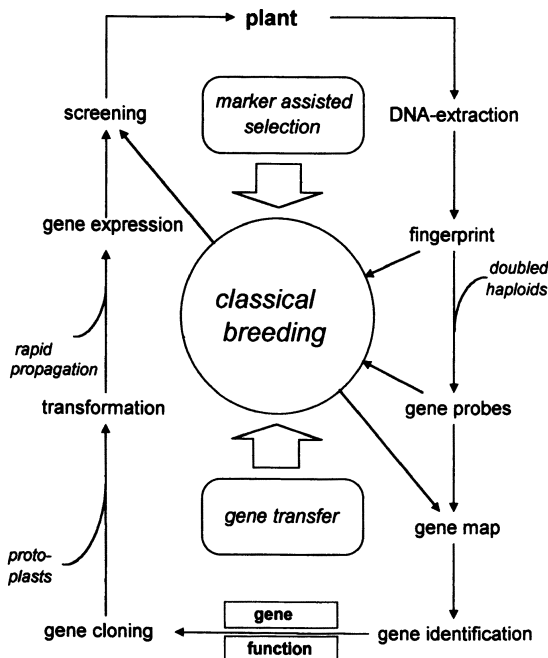


Fig. 1. Connections between different methodological tools: marker assisted selection gene function and gene transfer, together with overlap molecular genetics and breeding steps

appropriate virulences, cannot be differentiated based on their phenotype. Undoubtedly, molecular markers represent a useful tool for the breeder; their introgression into the breeding process will require, however, a revision of existing selection concepts. Figure 1 combines the MAS with the techniques necessary for gene identification and gene transfer. The dual purpose of this technique – for direct use in selection and for helping in gene isolation – is obvious. Fortunately, nature is rather conservative, visible by the high amount of similarity of R-gene sequences. Due to this synteny, it will not be necessary to start gene isolation and function analysis always right from the beginning but to make use of heterologous probes. The *mlo*-gene shows, however, that surprises can be expected.

## 8. Conclusions

The first phase of genome analysis was mainly characterized by joint efforts to construct comprehensive maps. During the next phase re-

search activities focused on the utilization of these maps for genetic localization of agronomic traits, e.g. by MAS, and for elucidating the function of the responsible genes. With respect to disease resistance, **about 150 alleles of major genes and QTLs have been identified.** There is preliminary evidence that some QTLs might represent less effective alleles of major genes because (1) many QTLs coincide with the genetic position of major genes, and (2) a series of QTL studies revealed one major QTL accounting for most of the genetic variability.

The physical isolation of resistance genes is a prerequisite for the functional analysis of the corresponding genes. Together with verification experiments by transformation, this elucidation will be a central research field. Although presently the most efficient gain of molecular technique is based in the area of MAS, the future will strengthen the transfer approaches. As soon as the gene function is understood, a third development will start, allowing the construction of resistance genes according to their specific needs. Presently only a few data exist on the verification of an anticipated gene function by gene transfer. In most cases, these examples come from the transfer of viral, phagous or bacterial genes. Transferring genes of one higher plant to another higher plant is still rare.

It can be expected that the speed of identifying resistance genes will increase significantly. One possible new strategy is **their identification by using the homology in the gene structure** for its identification. Leister et al. (1997) and Gebhardt et al. (1997) report on the production of R-gene homologues by a PCR approach that uses degenerate primers of conserved domains of NBR- and LRR-type resistance genes. Several primer sets were applied in PCR reactions with templates from rice or barley, genomic DNA or cDNA and tobacco or *Arabidopsis*, respectively. The PCR products were cloned and in both instances clones exhibiting significant homology were isolated. With this new approach **genome-wide mapping data of the rice R-gene homologues revealed several correlations to mapped resistance traits and lesion mimic loci as well as cosegregants of potato R-genes.**

Other approaches make use of the synteny, expecting that the conserved genomes have similar motives active in resistance (e.g. Killian et al. 1995). In most cases, such R-gene candidates have been found since their number was in most cases more than five. The proof which one of the five will be the correct one needs transformation techniques; since this is still difficult to routinely transfer numerous constructs, the answer is still missing. Progress will depend upon the genetic definition of the target gene. Particularly, induced and spontaneous mutants, as well as variants and mapping populations, will be of critical importance.

**Uncovering R-genes and their function relies also on good classical genetics and phenotypic characterizations.** A fruitful cooperation between classical and molecular genetics is the way to go. All successful crop varieties are selected for disease resistance, but up till now without knowing their exact molecular function. Since this strategy has already been quite successful, it can be expected that after understanding the R-gene



functions, man has for the first time the chance to be more efficient in plant protection than the concurring trial and error approach of pathogens.

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## Genetic Extranuclear Inheritance: Plastid Genetics

By Rudolf Hagemann, Monika M. Hagemann, and Ralph Block

### 1. Introduction

This chapter is a continuation of our chapter in *Progress in Botany* 57 (1996) and the previous articles in Volumes 47, 49, 51 and 55. In this chapter, we shall first report on the results of the complete sequencing of the plastid DNAs of several algae representing different taxa and shall work out the remarkable differences in the information content of these plastid genomes as compared with the coding capacity of the plastid DNAs of land plants which were dealt with in the previous volumes of *Progress in Botany*. Secondly, we will discuss recent results from genetic and molecular studies that have provided novel insights into the regulation of plastid gene expression and its interaction with the gene expression system in the nucleocytoplasmic compartment.

Before turning to these topics, we wish to draw the reader's attention to recent findings which *sensu stricto* do not belong to the field of extranuclear inheritance in eukaryotes, but nonetheless are of great relevance to this field:

Researchers interested in genetics and molecular biology of plastids have always taken a closer look at the genetic system of cyanobacteria, since – according to the endosymbiotic theory – ancient cyanobacteria are generally considered to be the ancestors of present-day plastids. Therefore, the report of the research team of Satoshi Tabata (Kazusa DNA Research Center, Chiba, Japan) about the **complete sequencing of the genome of the unicellular cyanobacterium *Synechocystis* sp.**, strain PCC6803 (3 573 470 bp) is of great interest to the community of organelle geneticists (Kaneko et al. 1996).

A total of 3168 potential protein-coding genes were identified on the *Synechocystis* genome. In addition, approximately 45 genes for structural RNAs (rRNA and tRNA genes) were found. The findings were that 145 genes (1.6%) are identical with previously reported *Synechocystis* genes, whereas 1257 (39.6%) and 340 (10.8%) genes show significant similarity to genes or hypothetical reading frames in other organisms respectively. The remaining 1426 (45%) genes have no apparent similarity to any genes in the sequence database. Among the protein-coding genes assigned, 128 are related to photosynthetic functions. A notable feature of this cyanobacterial genome is the presence of 99 open reading frames (ORFs) which exhibit similarity to transposase genes.

The long lasting discussion about the degree of homology between the cyanobacterial and plastid sequences as well as about the proportion of cyanobacterial genes that have been conserved during the evolutionary transition from the (hypothetical) cyanobacterial endosymbiont to present-day plastids, has now got a firm basis. An increasing number of plastid genomes have been completely sequenced during the past decade. With the sequence of the entire *Synechocystis* DNA, the complete genome of the cyanobacterial counterpart is now available for detailed comparison. All this information may have a profound influence on our understanding of the evolution of photosynthetic organelles.

## 2. Results and Implications of Complete Sequencing of Plastid Genomes of Several Algae

The sequencing of complete plastid genomes began with the sequence analysis of the plastid DNAs of several land plants (metaphytes): *Marchantia polymorpha* (1986), *Nicotiana tabacum* (1986), *Oryza sativa* (1989), *Epifagus virginiana* (1992), *Pinus thunbergii* (1994) and *Zea mays* (1995) (referenced in Hagemann and Hagemann 1994; Hagemann et al. 1996). In 1993, the total sequence of the plastid genome of *Euglena gracilis* (Euglenophyta) was reported (Hallick et al. 1993). Two years later, the complete sequences of the plastid genomes of three non-green algae representing widely different taxa were published: *Porphyra purpurea* (Rhodophyta: Rhodophyceae) by Reith and Munholland (1995); *Cyanophora paradoxa* (Rhodophyta: Glaucocystophyceae; cyanelles) by Stirewalt et al. (1995); and *Odontella sinensis* (Chromophyta: Bacillariophyceae, Diatomeae) by Kowallik et al. (1995).

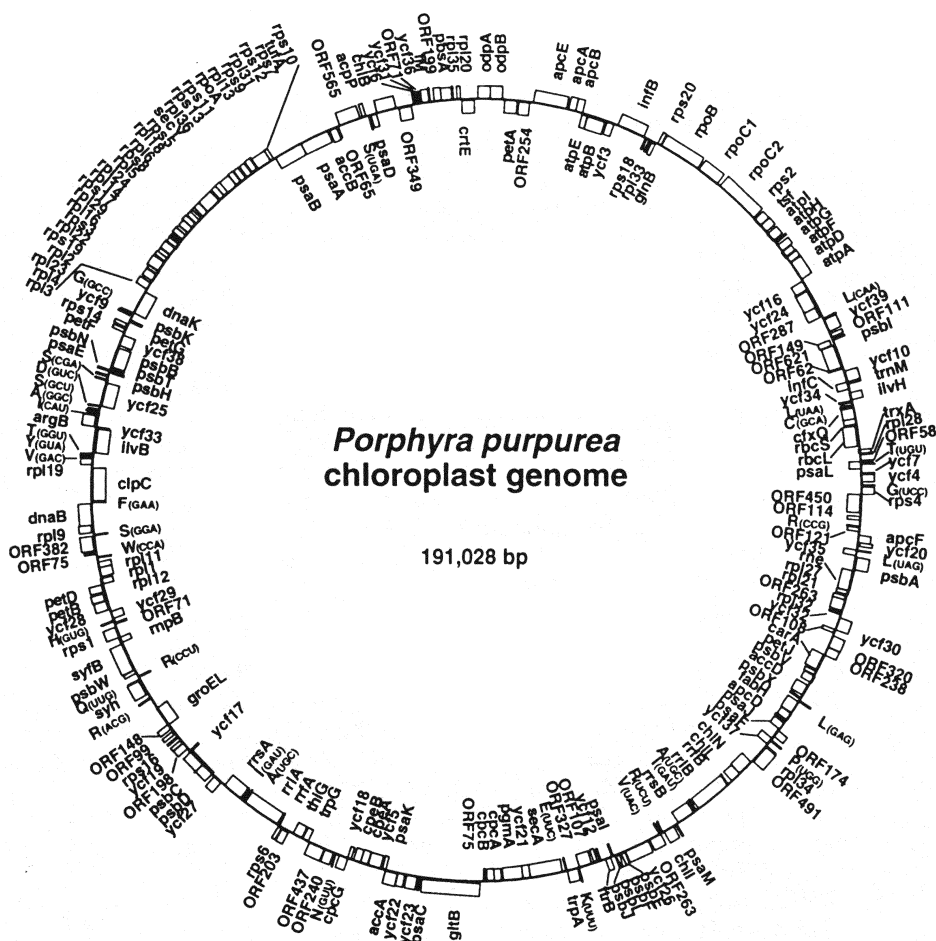
### a) *Euglena gracilis*

The *Euglena gracilis* plastid genome has several characteristics in which it differs from the typical plastid genomes of land plants: lack of inverted repeats, but presence of tandem repeats of the rDNA operon; numerous introns, many more than in any other known plastid genome, including group III introns which are specific for *Euglena*; presence of twintrons (a twintron is an intron interrupted by another intron). However, regarding the coding capacity the plastid genome of *Euglena* does not significantly differ from those of the land plants (Hallick et al. 1993); comp. physical map in *Progress in Botany* 57, p. 200.

In contrast, sequence analyses on the plastid DNAs of the three rhodophytic and chromophytic algae mentioned above unexpectedly revealed a much higher coding capacity.

### b) *Porphyra purpurea*

Exciting results were obtained from the analysis of the plastid DNA of the red alga *Porphyra purpurea*. Its circular plastid genome is 191028 bp in length and thus the largest plastid genome completely sequenced to date (Fig. 1). As most chloroplast DNAs, it has a low G+C content (33%) and contains two copies of the ribosomal RNA operon, here organized as direct repeats (inverted repeats in higher plants). The *Porphyra* plastid



genome contains 251 genes and open reading frames; this is more than double the number of plastid genes in metaphytes (land plants) (Reith and Munholland 1995).

Some of the most interesting features of the *Porphyra purpurea* plastid genome are:

- The presence of 53 genes encoding photosynthetic proteins: 11 genes for photosystem I, 16 for photosystem II, 8 for the ATPase, 10 for the phycobilisome, 4 for the cytochrome b<sub>6</sub>/f complex, 2 for Rubisco and 2 for mobile electron carriers (ferredoxin and cytochrome c<sub>553</sub>).
- It has 47 ribosomal protein genes: 28 for proteins of the large subunit, 19 for proteins of the small subunit.
- It has 35 tRNAs (complete set).
- It has 9 genes encoding proteins involved in gene expression: 2 initiation factors, 2 tRNA synthetases, 2 elongation factor subunits, a replication helicase subunit, the RNA component of RNase P and an RNase E homologue.
- It has 5 ORFs with probable functions as transcriptional regulatory proteins.
- It has 24 genes for biosynthetic functions, including synthesis of amino acids, fatty acids, pigments and thiamine.
- It has 65 ORFs (or *ycf*, hypothetical chloroplast reading frame), 19 of which are unique to *Porphyra purpurea*.
- It has no introns and there is no evidence for RNA editing.
- It has no genes (or pseudogenes) for subunits of the plastid NAD(P)H dehydrogenase complex.

Two main reasons account for the increased number of plastid genes in rhodophytes [compare Table 1 with Table 2 in Hagemann and Hagemann (1994) where the plastid genes of land plants are listed]:

1. Groups of plastid genes which are also present in land plants comprise significantly more genes in *Porphyra*, e.g. genes
  - for photosystem I: 11 in *Porphyra* instead of 6 in land plants
  - for ribosomal proteins: 47 instead of 22
  - for Rubisco: not only *rbcL*, but also *rbcS* is a plastid-encoded gene in red (and brown) algae.
2. More importantly, the algal plastid DNA contains entire groups of genes which are not present in plastid DNAs (but in the nuclear genome) of land plants, i.e. genes
  - for the control of DNA replication and gene expression and
  - for several biosynthetic functions.

Table 1 summarizes all genes and the corresponding gene products of the completely sequenced rhodophyte and chromophyte plastid genomes which are *not* found in metaphyte plastid DNAs.

**Table 1.** Genes of rhodophyte and chromophyte plastid genomes *not* found in metaphyte plastid genomes

Gene (and gene product)	Organisms
<i>Photosynthesis</i>	
<i>atpD</i> (ATP synthase CF <sub>1</sub> $\delta$ -subunit)	P, C, O
<i>atpG</i> (ATP synthase CF <sub>1</sub> $\gamma$ -subunit)	P, C, O
<i>cpcA</i> (phycocyanin $\alpha$ subunit)	P
<i>cpcB</i> (phycocyanin $\beta$ subunit)	P
<i>cpcG</i> (phycobilisome rod-core linker polypeptide)	P
<i>cpeA</i> (phycoerythrin $\alpha$ subunit)	P
<i>cpeB</i> (phycoerythrin $\beta$ subunit)	P
<i>petF</i> (ferredoxin)	P, C, O
<i>petJ</i> (cytochrome <i>c</i> <sub>553</sub> )	P, C, O
<i>petK</i> (cytochrome <i>c</i> <sub>550</sub> )	P, C
<i>psaD</i> (PSI, ferredoxin-binding protein, subunit II)	P, O
<i>psaE</i> (PSI, subunit IV, 18 to 20 kDa)	P, C, O
<i>psaF</i> (plastocyanin-binding protein, subunit III)	P, C, O
<i>psaK</i> [PSI, PSI-K polypeptide ('P37')]	P
<i>psaL</i> (PSI reaction centre subunit XI)	P, O
<i>psbU</i> (9- or 12-kDa protein of oxygen-evolving complex)	P
<i>psbV</i> [cytochrome <i>c</i> <sub>550</sub> (oxygen-evolving compartment)]	P, C, O
<i>psbW</i> [PSII protein W (13 kDa)]	P, C, O
<i>psbX</i> [PSII protein X (4.1 kDa)]	P, C, O
<i>rcbS</i> (ribulose-bisphosphate carboxylase, small unit)	P, C, O
<i>Gene expression</i>	
<i>dnaB</i> (replication helicase subunit)	P, O
<i>dnaK</i> (hsp 70-type chaperone)	P, C, O
<i>infB</i> (initiation factor 2)	P
<i>infC</i> (initiation factor 3)	P
<i>rne</i> (RNase E)	P
<i>rnpB</i> (RNA component of RNase P)	P, C
<i>rpl1</i> (ribosomal protein L1)	P, C, O
<i>rpl3</i> (ribosomal protein L3)	P, C, O
<i>rpl4</i> (ribosomal protein L4)	P
<i>rpl5</i> (ribosomal protein L5)	P
<i>rpl6</i> (ribosomal protein L6)	P, C, O
<i>rpl9</i> (ribosomal protein L9)	P
<i>rpl11</i> (ribosomal protein L11)	P, C, O
<i>rpl12</i> (ribosomal protein L12)	C, O
<i>rpl13</i> (ribosomal protein L13)	P, O
<i>rpl18</i> (ribosomal protein L18)	P, C, O
<i>rpl19</i> (ribosomal protein L19)	P, C
<i>rpl24</i> (ribosomal protein L24)	P
<i>rpl27</i> (ribosomal protein L27)	P, O
<i>rpl28</i> (ribosomal protein L28)	P
<i>rpl29</i> (ribosomal protein L29)	P, O

**Table 1** (continued)

Gene (and gene product)	Organisms
<i>prl31</i> (ribosomal protein L31)	P, O
<i>rpl34</i> (ribosomal protein L34)	P, C, O
<i>rpl35</i> (ribosomal protein L35)	P, C, O
<i>rps1</i> (ribosomal protein S1)	P
<i>rps5</i> (ribosomal protein S5)	P, C, O
<i>rps6</i> (ribosomal protein S6)	P, C, O
<i>rps9</i> (ribosomal protein S9)	P
<i>rps10</i> (ribosomal protein S10)	P, C, O
<i>rps13</i> (ribosomal protein S13)	P, C, O
<i>rps17</i> (ribosomal protein S17)	P, C, O
<i>rps20</i> (ribosomal protein S20)	P, C, O
<i>syfB</i> (phenylalanine tRNA synthetase)	P
<i>syh</i> (histidine tRNA synthetase)	P
<i>trnL</i> (GAG) (transfer RNA leu)	P
<i>trnR</i> (CCU) (transfer RNA arg)	P, O
<i>trnS</i> (CGA) (transfer RNA ser)	P, O
<i>trxA</i> (EnvZ-like regulator protein)	P
<i>trxB</i> (putative transcriptional regulatory protein)	P, C
<i>trxC</i> (putative transcriptional regulatory protein)	P
<i>trxD</i> (putative transcriptional regulatory protein)	P
<i>trxE</i> (putative transcriptional regulatory protein)	P, C, O
<i>tsf</i> (elongation factor Ts)	P
<i>tufA</i> (elongation factor Tu)	P, C, O
<b>Biosynthesis</b>	
<i>accA</i> (acetyl-CoA carboxylase carboxytransferase, $\alpha$ subunit)	P
<i>accB</i> (acetyl-CoA carboxylase biotin carboxyl carrier protein subunit)	P
<i>accD</i> (acetyl-CoA carboxylase carboxytransferase $\beta$ subunit)	P
<i>acpP</i> (acyl carrier protein)	P, C, O
<i>apcA</i> (allophycocyanin $\alpha$ subunit)	P, C, O
<i>apcB</i> (allophycocyanin $\beta$ subunit)	P
<i>apcD</i> (allophycocyanin $\gamma$ subunit)	P
<i>apcE</i> (phycobilisome core linker polypeptide)	P
<i>apcF</i> (allophycocyanin B18 subunit)	P
<i>argB</i> (acetylglutamate kinase)	P
<i>carA</i> (carbamoyl phosphate synthase small subunit)	P
<i>chlB</i> (protochlorophyllide reductase ChlB chain)	P
<i>chlI</i> (magnesium chelate subunit)	P
<i>chlL</i> (protochlorophyllide reductase iron-sulphur ATP-binding protein)	P
<i>chlN</i> (protochlorophyllide reductase ChlN chain)	P
<i>crtE</i> (geranylgeranyl pyrophosphate synthase)	C
<i>fabH</i> ( $\beta$ -ketoacyl-acyl carrier protein synthase III)	P
<i>ftb</i> (ferredoxin-thioredoxin reductase $\beta$ subunit)	P
<i>ftsW</i> [putative cell (organelle) division protein]	
<i>glnB</i> (nitrogen regulatory protein PII)	P
<i>gltB</i> [glutamate synthase (GOGAT)]	P
<i>hemA</i> (5-aminolevulinic acid synthase)	C
<i>hesB</i> (protein involved in nitrogen assimilation)	P

Table 1 (continued)

Gene (and gene product)	Organisms
<i>hisH</i> (histidinol-phosphate aminotransferase)	C
<i>hisP</i> (histidine transport ATP-binding protein)	C
<i>ilvB</i> (acetohydroxyacid synthase large subunit)	P
<i>ilvH</i> (acetohydroxyacid synthase small subunit)	P
<i>nadA</i> (quinolinate synthase)	C
<i>odpA</i> (pyruvate dehydrogenase E <sub>1</sub> component, $\alpha$ subunit)	P
<i>odpB</i> (pyruvate dehydrogenase E <sub>1</sub> component, $\beta$ subunit)	P
<i>pbsA</i> (heme oxygenase)	P
<i>pgmA</i> (phosphoglycerate mutase)	P
<i>preA</i> (prenyl transferase)	P, C
<i>thiG</i> (thiG protein, thiamine biosynthesis)	P
<i>trpA</i> (tryptophan synthase $\alpha$ subunit)	P, A
<i>trpG</i> (anthranilate synthase component II)	P, C
<i>Miscellaneous</i>	
<i>cfxQ</i> (involved in Rubisco-expression)	P, O
<i>clpB</i> (clp protease subunit)	P
<i>clpC</i> (clp protease ATP-binding subunit)	P
<i>dnaK</i> (hsp70-type chaperone)	P, C, O
<i>ftsH</i> (protease)	P, O
<i>groEL</i> (60-kDa chaperonin)	P, C, O
<i>groES</i> (10-kDa chaperonin)	C
<i>secA</i> (preprotein translocase subunit)	P, O
<i>secY</i> (preprotein translocase subunit)	P, C, O
<i>trxA</i> (thioredoxin)	P

P, *Porphyra purpurea*; C, *Cyanophora paradoxa*; O, *Odontella sinensis*.

In addition to genes listed above, at least 32 hypothetical reading frames (*ycfs*) have been found, the gene product of which is still unknown.

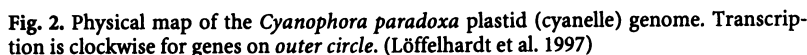
Lists of plastid-encoded genes from algae, plastid genomes of which are not fully sequenced, are given in Löffelhardt and Bohnert (1994b) and Reith (1995).

Data compiled in this list have been taken from papers of Löffelhardt and Bohnert (1994b), Reith (1995), Reith and Munholland (1995), Kowallik et al. (1995) and Stirewalt et al. (1995).

### c) *Cyanophora paradoxa*

*Cyanophora paradoxa* contains a special type of plastids termed cyanelles. Cyanelles were initially considered to be relatively recent cyanobacteria-like endosymbionts. Like cyanobacteria, they are surrounded by a peptidoglycan wall. They also resemble cyanobacteria in their pigment composition and in possessing a carboxysome-like structure. *Cyanophora paradoxa* is the best-investigated member of the Glaucocystophyceae. Taxonomically, the Glaucocystophyceae form together with the Rhodophyceae (e.g. *Porphyra*) and the Cyanidiophyceae (or pre-rhodophytes) the division of Rhodophyta (Reith 1995).

The circular cyanelle genome is 135599 bp in length (Stirewalt et al. 1995). It has a G+C content of 30.4% and carries two inverted repeats (IR<sub>A</sub> and IR<sub>B</sub>) of 11285 bp each a large single copy (LSC) region of 94946 bp and a small single copy (SSC) region of 18083 bp (Fig. 2). The





cyanelle genome contains 192 genes and open reading frames. Although the cyanelle DNA is somewhat smaller than the plastid DNA of tobacco, it nevertheless encodes 30% more genes than the tobacco plastid genome.

The genes present in the cyanelle DNA but not in the plastid genome of land plants are compiled in Table 1. A few additional characteristics of the cyanelle DNA are noteworthy:

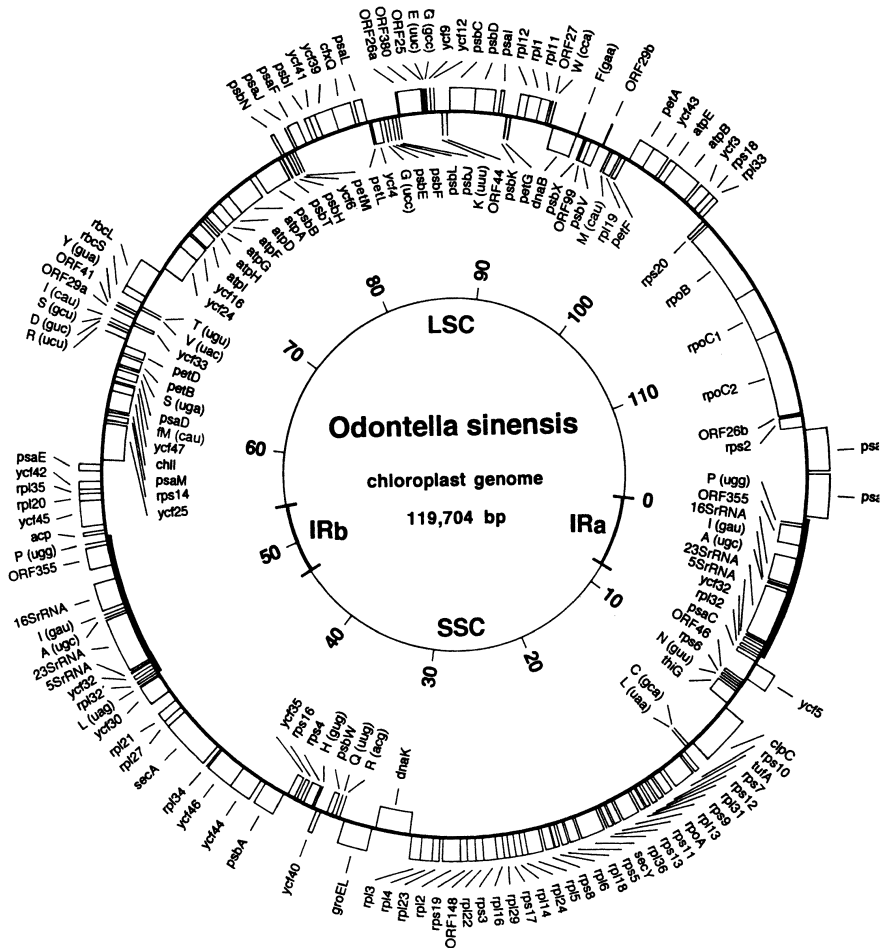
- presence of a single group I intron located in *trnL* (UAA) in a position that is conserved in many cyanobacteria and nearly all plastid DNAs
- complete absence of group II introns
- no *ndh* genes or pseudo-*ndh* reading frames (encoding subunits of a putative plastid-localized NAD(P)H dehydrogenase) (for more detailed information see Stirewalt et al. 1995).

In summary, the information obtained from the analysis of the cyanelle genome of *Cyanophora paradoxa* provides evidence for cyanelles as true plastids. As all plastids, cyanelles are derived from endosymbiotic cyanobacteria and have lost more than 90% of the genetic information of their cyanobacterial ancestor. However, they can still be regarded as 'molecular fossils' since they retained the prokaryotic (peptidoglycan) wall. Regarding their coding capacity, they resemble the Rhodophyceae (e.g. *Porphyra*) and Chromophyta (e.g. *Odontella*) and, therefore, most likely do not originate from a distinct endosymbiosis event.

#### d) *Odontella sinensis*

The plastid genome of the centric diatom *Odontella sinensis* (Chromophyta) was completely sequenced by Kowallik et al. (1995). The circular molecule comprises 119704 bp (Fig. 3). An inverted repeat (IR) of 7725 bp containing the rRNA operon separates a large single copy region of 65346 bp from the small single copy region of 38908 bp. The *Odontella* plastid DNA has a G+C content of 31.8%, and 174 genes and open reading frames have been identified, nine of which are duplicated within the inverted repeat. In four cases, codon GUG is used as initiation codon instead of the standard codon AUG. In Table 1, the plastid genes of *Odontella* which are not found in the plastid genomes of land plants are listed.

The *Odontella sinensis* plastid genome does not contain any group I or group II introns. Like the plastid DNAs of other non-green algae, the *Odontella* plastid genome lacks all *ndh* genes. Interestingly, it shows gene overlapping for as many as four pairs of genes (*rpl14/rpl23* 8 bp; *ycf24/ycf6* 1 bp; *psbD/psbC* 53 bp; *atpF/atpD* 4 bp, Kowallik et al. 1995).



### e) *Evolutionary Implications*

Compelling molecular evidence has confirmed that both plastids and mitochondria originated as bacterial endosymbionts. However, it has still been a matter of debate whether the primary incorporation of a cyanobacterium into a pre-eukaryotic cell occurred only once in evolution (**monophyletic origin** of plastids) or several times (**polyphyletic origin**).

Polyphyletic origin hypotheses mainly rely on differences in photosynthetic pigment composition. However, the sequence data obtained from the analysis of algal plastid genomes as well as the construction of phylogenetic trees provide good arguments for a *monophyletic origin of all present-day plastids* (Kowallik 1992, 1994; Gray 1993; Reith 1995). In accordance with the endosymbiotic theory, there was only one process of endosymbiotic incorporation of an ancestral cyanobacterium which then led to the plastids in all plant lineages. This endosymbiont lost in the course of conversion into plastids most of its genetic material and information; only about 2 to 5% of the cyanobacterial genome was retained. Plastid genomes with an increased coding capacity (e.g. *Porphyra purpurea*) may typify a primitive type of plastids where less genes were evolutionarily transferred to the nucleus. The plastid genomes of land plants (metaphytes) may represent the most derived type of present-day plastids and thus the temporary end of plastid evolution.

### 3. Regulation of Gene Expression in Plastids

#### a) Transcription of Chloroplast Genes

In view of the accumulating evidence for an endosymbiotic origin of plant organelles, the identification of plastid genes encoding subunits of an *E. coli*-like DNA-dependent RNA polymerase about 10 years ago fitted well into the emerging picture of a prokaryotic gene expression system in plastids (for review see Igloi and Kössel 1992). The *E. coli* RNA polymerase core enzyme is encoded by three genes (*rpoA*, *rpoB*, *rpoC*), the products of which ( $\alpha$ ,  $\beta$  and  $\beta'$ ) assemble in a 2:1:1 stoichiometry. The structure of the core polymerase can thus be defined as  $\alpha_2\beta\beta'$ . The subunit structure of the **chloroplast enzyme** is homologous to its bacterial counterpart except that the N- and C-terminal portions of the  $\beta'$  subunit are encoded by separate genes (*rpoC1* and *rpoC2*, respectively) and thus are no longer physically linked (the two polypeptide chains were termed  $\beta'$  and  $\beta''$ ). Interestingly, such a  $\alpha, \beta\beta''$  composition of the RNA polymerase is also observed in cyanobacteria (Xie et al. 1989), the presumptive ancestors of present-day chloroplasts. The split of the *rpoC* gene into two pieces is therefore likely to predate the primary endosymbiosis event. It was recently demonstrated that the *rpoC1* and *rpoC2* gene products can be reconstituted in *E. coli* RNA polymerase and functionally replace the *E. coli*  $\beta'$  subunit (Severino et al. 1996). RNA accumulation data and Western blot analyses with antibodies raised against individual subunits confirmed the active expression of the plastid-encoded *rpo* genes. In addition, the chloroplast RNA-polymerizing ac-

tivity was biochemically characterized both in its soluble and in its DNA-bound form (Igloi and Kössel 1992).

Auxiliary protein factors ( $\sigma$  factors) are known to impose promoter specificity onto the bacterial RNA polymerase core enzyme. The presence of  $\sigma$ -like factors in chloroplasts was demonstrated by the identification of plastid protein fractions that enhance binding of the *E. coli* core polymerase to chloroplast promoters and increase transcriptional activity in vitro (Tiller et al. 1991; Tiller and Link 1993). Usage of distinct  $\sigma$ -like factors was proposed to account for plastid type-specific transcription patterns. In addition, the regulation of a  $\sigma$  factor activity by reversible phosphorylation and dephosphorylation may contribute to the light-regulated transcription of chloroplast genes (Tiller and Link 1993). Recently the gene for a plastid-localized  $\sigma^70$ -like factor was cloned from the red alga *Cyanidium caldarium* (Liu and Troxler 1996; Tanaka et al. 1996). Further molecular studies of this and other putative members of the family of plastid  $\sigma$ -like factors will certainly provide novel insights to the structure and function of the plastid transcriptional apparatus.

Several reports have suggested the existence of a second distinct transcription system in plastids of higher plants. This system was proposed to rely on a **nuclear-encoded RNA polymerase activity that is targeted to the chloroplast compartment**. Circumstantial evidence for such a second RNA polymerase has come from genetic, molecular biological and biochemical analyses:

1. Promoter analyses revealed a subset of plastid genes lacking recognizable  $\sigma^70$ -like promoters (Gruissem et al. 1986; Klein et al. 1992; Vera and Sugiura 1995; Vera et al. 1996). This suggests that some genes are not efficiently transcribed by the plastid-encoded (*E. coli*-like) RNA polymerase.
2. Active transcription was shown to occur in plastids of the non-photosynthetic plant *Epifagus virginiana*. This root parasite harbours a minimal chloroplast genome lacking nearly all photosynthesis-related genes and also all of the RNA polymerase subunit genes (Mordon et al. 1991). Uncertainty remains, however, with respect to a possible gene transfer of the formerly plastid-encoded RNA polymerase genes to the nucleus of *Epifagus*.
3. Ribosomes were shown to be completely absent from plastids of the barley mutant *albostrans* (Hagemann und Scholz 1962) suggesting that these organelles are deficient in translation of all plastid-encoded genes (Börner, Schumann and Hagemann 1976; Hess et al. 1993, 1994). Hence, also the subunits of the *E. coli*-like RNA polymerase should not be present in the mutant plastids. Nevertheless, a subset of plastid genes was shown to be actively transcribed in *albostrans* (Hess et al. 1993).
4. Biochemical analyses have revealed at least two separable RNA polymerase activities in isolated spinach and mustard chloroplasts (Lerbs-Mache 1993; Pfannschmidt and Link 1994). Whereas one of the activities exhibited sensitivity to the prokaryotic RNA polymerase inhibitor rifampicin, the second activity was found to be resistant. In addition, the relative activities of the two RNA polymerizing complexes are subject to changes in response to light and developmental programs.

Two recent reports unambiguously demonstrated that there is indeed a nuclear-encoded RNA polymerase present in higher plant plastids. Deletion of the *rpoB* gene from the tobacco chloroplast genome using plastid transformation abolished all transcriptional activity by the plastome-encoded (*E. coli*-like) RNA polymerase (Allison et al. 1996). Analysis of residual RNA synthesis in the mutant plastids revealed the existence of a second presumably nuclear-encoded RNA polymerase which does not utilize *E. coli*-like promoters. This novel RNA polymerase activity seems to preferentially transcribe plastid genetic system genes rather than photosynthesis genes. Although the generated  $\Delta rpoB$  mutant plants are photosynthetically incompetent, transcription by the second RNA polymerase appears to be sufficient for plastid maintenance and plant development (Allison et al. 1996).

In a similar study, transcription in tissue culture-induced *rpo* gene deletion mutants was investigated. Cell and tissue culture conditions are known to induce large-scale deletions in cereal chloroplast DNAs. A mutant rice cell line was identified that harbours only a residual chloroplast genome of 19.3 kb covering a tRNA-rich region of the wild-type genome (Chiba et al. 1996). In this line, all RNA polymerase genes are absent from the chloroplast DNA. Nonetheless, transcription of the tRNA genes was clearly detectable in mutant plastids, again suggesting that there is an additional RNA polymerase activity which is not encoded by the chloroplast genome (Chiba et al. 1996).

The existence of two plastid RNA polymerase with largely different promoter specificities now raise the attractive possibility that the nuclear-encoded polymerase is the predominant transcriptional activity early in plastid biogenesis. It actively transcribes genetic system genes, and among those also the genes for the plastid-encoded RNA polymerase subunits. Once switched on, the *E. coli*-like enzyme becomes the major RNA synthesizing activity by transcribing all photosynthesis-related genes and to a certain extent also genetic system genes. Thus, the polymerase switch may be a crucial step in the differentiation pathway from proplastids to chloroplasts.

## b) Transcriptional Regulation

The general transcription rate of chloroplast genes is mainly controlled by promoter strength (for review see, e.g., Gruissem and Tonkyn 1993). Transcriptional run-on assays performed with isolated barley plastids revealed that the transcription rates of the 15 plastid genes examined vary more than 300-fold (Rapp et al. 1992). In addition to differential promoter strength, several factors are known to modulate transcription in a tissue-specific and/or developmental stage-specific manner:

- Promoter type and RNA polymerase usage. Transcription of a number of chloroplast genetic system genes is driven by multiple promoters. Interestingly, in many cases one and the same gene or operon is preceded by both a  $\sigma^{70}$ -like promoter and an alternative non-*E. coli*-like promoter most likely used by the nuclear-encoded plastid RNA polymerase (Vera and Sugiura 1995; Vera et al. 1996). This scenario offers an attractive opportunity for the fine-tuning of chloroplast transcription by the interplay of two polymerases and two promoters of defined strength.
- $\sigma$  Factor usage (see Section 3.a.).
- Putative non- $\sigma$ -like transcription factors. In vitro and in vivo studies using the plastid *psbD/C* operon have suggested that the light-regulated transcription of chloroplast genes is mediated by a class of transcription factors with different properties from bacterial type  $\sigma$  factors (Wada et al. 1994; Allison et al. 1997). These factors seem to be present in illuminated chloroplasts only and bind to DNA elements upstream of the -35/-10 core promoter sequences. The molecular characterization of these transcription factors should help elucidate their mode of interaction with the RNA polymerase as well as the details of the transduction pathway of the light signal.

### c) Post-transcriptional Regulation

Compared with the role of transcriptional regulation, a much greater contribution to the control of plastid gene expression comes from post-transcriptional processes (Deng and Gruissem 1987; for review see, e.g. Gruissem and Tonkyn 1993; Sugita and Sugiura 1996). Post-transcriptional regulatory mechanisms in plastids act at four control levels: (1) transcript processing, (2) RNA turnover, (3) translation and (4) protein stability and degradation. In this chapter, we shall concentrate on the first three control mechanisms. For a comprehensive view of post-translational regulation in chloroplasts, the reader is referred to an excellent review published recently (Adam 1996).

#### $\alpha$ ) RNA Processing and Stability

The regulation of transcript processing and RNA stability is known to be an important determinant for gene expression in both prokaryotic and eukaryotic systems. Plastid primary transcripts undergo a series of RNA maturation processes: cleavage of polycistronic into monocistronic mRNAs, 5' and 3' terminal processing, group I and group II intron splicing and RNA editing. All of these processing steps can be subject to changes in response to developmental programs or environmental fac-

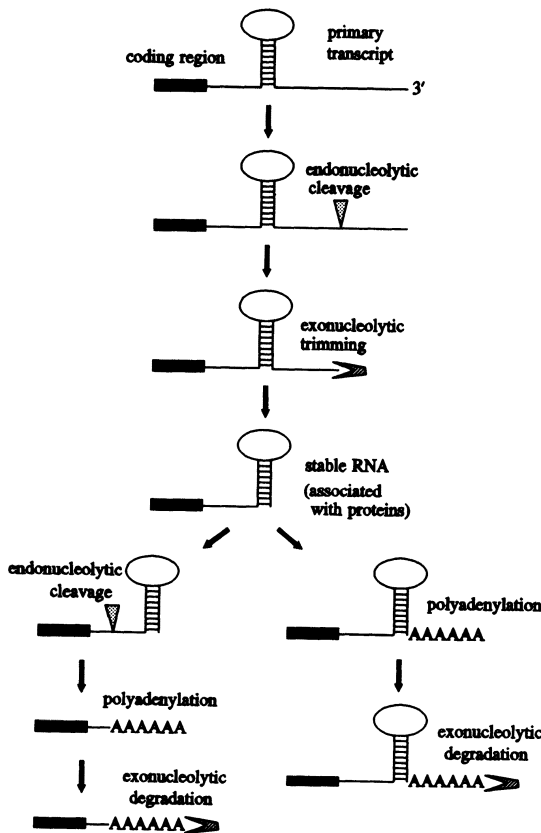
tors and can, therefore, potentially influence the stability or translatability of plastid transcripts.

Generally, the transcription rates of chloroplast genes do not strictly correlate with the steady-state RNA levels (Rapp et al. 1992) pointing to differential RNA stability as an important factor in plastid gene expression. The relative mRNA stabilities were measured for several barley plastid genes and found to vary at least 30-fold (Rapp et al. 1992). In addition, the stability of one and the same transcript can vary in a developmental stage-dependent manner. For example, the developmental transition from proplastids to chloroplasts is accompanied by no or only minor changes in the relative transcription rates of many plastid-encoded genes. However, several mRNAs accumulate rapidly with the onset of chloroplast biogenesis, most probably due to an increase in their half-life time (Mullet 1988; Grussem and Tonkyn 1993).

The main transcript stabilizing mechanisms known to operate in eukaryotic systems are absent from the plastid compartment: chloroplast primary transcripts do not undergo 5' modification (capping) and are not stabilized by 3' poly(A)-tail addition. What then are the factors determining plastid transcript stability?

Several plastid transcripts show multiple 5' ends resulting from endonucleolytic cleavage within the 5' untranslated region of the mRNA. At present, it is not known whether differential 5' maturation is a regular control mechanism modulating either RNA stability or translatability of the message. Endonucleolytic cleavage is also involved in plastid mRNA 3'-end processing (Hayes et al. 1996; Yang et al. 1996). In the absence of efficient transcription termination, correct post-transcriptional 3'-end formation is a crucial step in the production of stable plastid RNAs. An endonucleolytic cleavage event downstream of a characteristic stem-loop structure in the 3' untranslated region of the primary transcript initiates 3'-processing (Fig. 4). The sequence in between this secondary structure element and the cleavage site is subsequently removed by an exoribonuclease (100 RNP; Hayes et al. 1996). The trimmed 3'-end remains associated with (nuclear-encoded) RNA-binding proteins (Schuster and Grussem 1991; Hayes et al. 1996) and protects the mRNA from degradation. Several lines of evidence support the assumption that these RNA-binding proteins play an important role in the control of plastid mRNA turnover: their mRNA and protein accumulation is subject to developmental regulation (Schuster and Grussem 1991) and their activity is modulated by protein phosphorylation (Lisitsky and Schuster 1995).

It was recently shown that plastid mRNA degradation is associated with polyadenylation (Lisitsky et al. 1996; Kudla et al. 1997; Fig. 4). Apparently 3' poly(A) addition renders the mRNA susceptible to a 3' → 5' exonucleolytic activity. Remarkably, polyadenylation not only triggers degradation of RNA molecules devoid of the 3' stem-loop structure (due to an upstream endonucleolytic cleavage event as the first step in the degradation pathway) but also can trigger RNA decay in the presence of the 3' secondary structure which then is no longer protective against the exoribonuclease (Fig. 4; Kudla et al. 1997). The extent of polyadenylation



**Fig. 4.** Model for 3'-end processing and RNA degradation in plastids. In absence of efficient transcriptional terminator structures, read-through transcription leads to production of mRNAs with extended 3'-ends. A protein complex containing both endoribonuclease activity (Yang et al. 1996) and an exoribonuclease (Hayes et al. 1996) degrades sequences downstream of stem-loop structure and thus produces stable mRNA. Mature 3'-ends remain associated with protein complex binding to stem-loop structure and still containing nucleolytic activities. Formation of this ribonucleoprotein complex prevents further degradation of transcript and thus provides stable substrates for translation by chloroplast ribosomes, mRNA decay is initiated by alternative mechanisms (Kudla et al. 1997): (1) endonucleolytic cleavage upstream of stem-loop structure followed by 3' polyadenylation which in turn elicits degradation by a 3' → 5' exoribonuclease activity; or (2) polyadenylation downstream of stem-loop structure (possibly facilitated by dissociation of 3'-ribonucleoprotein complex) and subsequent exonucleolytic degradation




of photosynthesis gene transcripts was found to increase in the dark (Kudla et al. 1997) suggesting that, in the absence of light, up-regulation of the polyadenylation activity promotes mRNA degradation. Interestingly, polyadenylation was also shown to be involved in mRNA decay in *E. coli* (Xu and Cohen 1995). This finding as well as the strong similarity of the *E. coli* and plastid RNA-degrading enzymes (Hayes et al. 1996) point to evolutionarily well-conserved mechanisms of RNA metabolism in prokaryotes and plastids.

All of the other plastid RNA-processing activities, such as processing of polycistronic into monocistronic mRNAs (Haley and Bogorad 1990; Barkan et al. 1994), splicing (Barkan 1989) and RNA editing (Bock et al. 1993), can also vary during plastid development, in different tissues or in response to changes in environmental conditions. The biological significance of these variations and their role in RNA metabolism as well as in the differential regulation of plastid gene expression is, however, less clear.

### β) Translational Regulation

Translational regulation appears to be the main control level for the expression of plastid-encoded photosynthesis genes. mRNAs for most photosynthetic proteins are stable and accumulate in the dark as well as in non-photosynthetic tissues, although the encoded proteins are not synthesized. Recent data indicate that light exerts the control of translation initiation through the redox status of the chloroplast. How is plastid translation coupled to a redox reaction, i.e. to light-driven electron transfer? Based on in vitro binding studies involving the *Chlamydomonas psbA* mRNA and putative translational activator proteins, Mayfield and colleagues have suggested a model for the redox regulation of chloroplast translation (Danon and Mayfield 1994). In this model, light modulates the translation of chloroplast mRNAs by changing the RNA-binding properties of translation factors (Fig. 5). The increasing reduc-



**Fig. 5.** Model for redox regulation of chloroplast translation. Illumination of plant cells (*right panel*) turns on light reaction of photosynthesis which generates electrons to reduce pool of electron carrier ferredoxin (*Fd*). Reduced ferredoxin is capable of reducing disulphide bonds of thioredoxin known as redox signalling protein in many systems. Reduced thioredoxin transduces redox signal to regulatory mRNA-binding proteins acting as (subunits of) translational activators. Reduction of disulphide bonds in translational activator induces conformational change and facilitates binding to structured *cis*-element in 5' untranslated region of mRNA. Assembly of mRNA-translational activator protein complex then allows for translation initiation. In the dark (*left panel*), ferredoxin pool remains in its oxidized state, thus preventing reduction of thioredoxin. Oxidized translational activator proteins adopt a conformation which is not capable of ribonucleoprotein complex formation with 5' untranslated of the mRNA. Hence, lack of initiation complex formation prevents translation in the dark

ing power generated by photosynthesis in the light reduces the redox carrier ferredoxin which in turn reduces thioredoxin. The reduced thioredoxin then reduces regulatory disulphide bonds of translational activator proteins, thus allowing for their binding to 5' untranslated regions of plastid mRNAs and switching on translation (Danon and Mayfield 1994; Levings and Siedow 1995; Fig. 5). Remarkably, formation of the

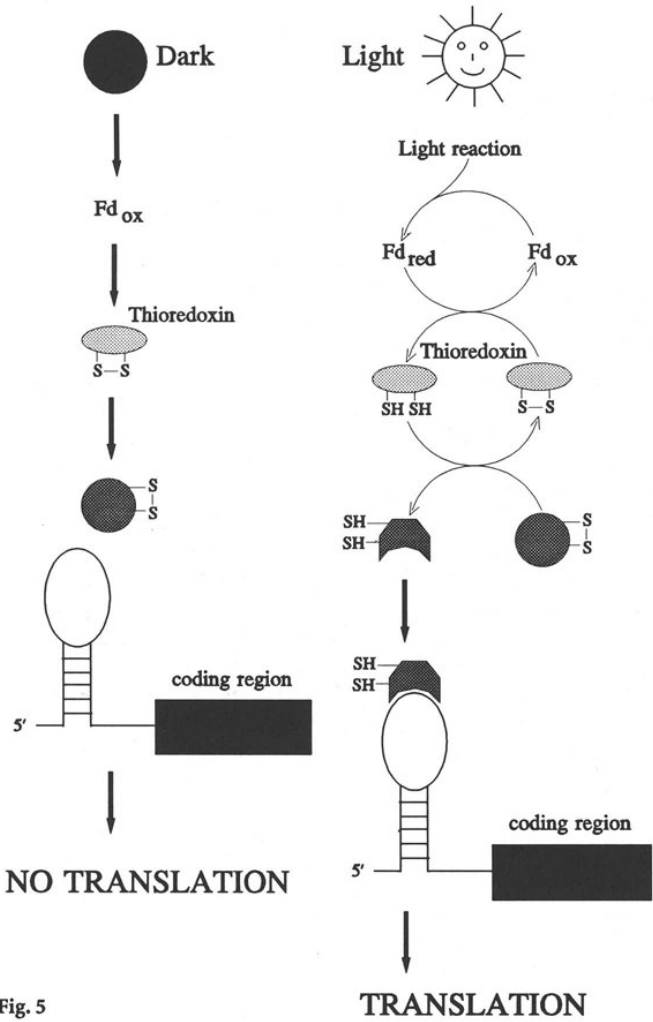


Fig. 5

mRNA-translational activator protein complex can be reconstituted in vitro by the vicinal dithiol reductant dithiothreitol which seems to efficiently replace the redox signal used in vivo (Danon and Mayfield 1994).

According to this model, electron carriers such as ferredoxin and thioredoxin act as transducers of the redox signal generated by the light-reaction of photosynthesis. This may provide the chloroplast with an efficient mechanism to adjust the synthesis of photosynthetic proteins to fluctuating light levels. In view of the risk of uncontrolled electron and free radical generation by photosynthesis, a rapid switch from a translationally inactive plastid RNA pool to a high rate of photosynthetic protein synthesis is certainly an important factor for the plant's fast response to environmental changes.

It is well-established that *chloroplast signals* also regulate the transcription of certain nuclear-encoded chloroplast genes (for review see, e.g., Susek and Chory 1992). Photosynthetically active chloroplasts seem to be required for active transcription of nuclear-encoded photosynthesis-related genes, such as *rbcS* and *cab* (encoding the small subunit of ribulose biphosphate carboxylase and the chlorophyll a/b-binding protein of the photosystem II light harvesting complex, respectively). In addition, the transcription of genes for several extraplastidic activities is dependent on functional chloroplasts, e.g. the nitrate reductase gene (encoding a cytosolic enzyme) as well as the peroxisomal glycolate oxidase and hydroxypyruvate reductase genes. Nuclear mutations have been described that disrupt this signal transduction pathway from the chloroplast to the nucleus (Susek et al. 1993). In these mutants, *rbcS* and *cab* can be efficiently expressed in the absence of photosynthesis and chloroplast development. However, all of the nuclear mutations seem to affect late steps in the signalling cascade from the chloroplast to the nucleus. This primary signal (i.e. the long-sought 'plastidic factor') is most probably not a nuclear-encoded gene product. Recent data concerning *cab* gene expression in the green alga *Dunaliella tertiolecta* highlight the possibility that reduction/oxidation of the plastoquinone pool mediates the flow of regulatory information from the chloroplast to the nucleus (Escoubas et al. 1995). Thus, the redox state of the chloroplast seems to be a universally used signal and may not only regulate plastid genes at the level of translation but also coordinate nuclear and plastid gene expression.

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## **Molecular Cell Biology: Different Transcriptional Activities in the Nucleus**

By Ulrike Zentgraf, Riccardo Velasco, and Vera Hemleben

### **1. Introduction**

Plant growth and development is dependent on numerous environmental and endogenous factors. Plant cells are able to respond to different signals with very precise reactions using different complex signal transduction pathways. A multiplicity of transmitting agents are involved in these complex processes which start with signal perception by receptor molecules, moving on to signal transduction in the cell to the nucleus, resulting in the expression of specific genes and ending with a response to the signal which can be local or systemic. In most cases, the result of activating such a signal transduction pathway is the stimulation or repression of the expression of one or several genes coding for products involved in the cell answer. Therefore, after the perception of the signal by exogenous or endogenous receptors the signal has to be transmitted to the nucleus (for review see Zentgraf and Hemleben 1996). The activation of specific genes is achieved by elevating the rate of transcription initiation at the respective promoters. The basal transcription initiation complex which is formed by ubiquitous transcription factors and RNA polymerase is complemented by specifically regulated *trans*-factors interacting with defined *cis*-elements. The concentration of these specific *trans*-factors, their compartmentalization, their stage of activity and their multimerization determine the transcription rate.

Transcription of the three different classes of RNA polymerases generally existing in eukaryotic nuclei takes place in different compartments of the nucleus: RNA polymerase I (Pol I), responsible for the transcription of the 18S, 5.8S and 25S/28S ribosomal RNA genes, is acting in the nucleolus, whereas the RNA polymerase II (Pol II) transcribing the polypeptide coding genes and some small nuclear RNA (snRNA or U-snRNA) genes and RNA polymerase III (Pol III) transcribing the 5S ribosomal RNA genes, transfer RNA (tRNA) genes and other snRNA genes fulfil their function in other domains of the nucleus (for review see Spector 1993). Establishment of these compartments within the nucleus and its implications for transcriptional regulation is still a question of cell biology which is not answered at all.



However, not only the concentration and DNA-binding of the *trans*-factors to their *cis*-elements and their interaction with the basal transcription machinery are involved in transcriptional regulation. Many other aspects have to be taken into account for the modulation of gene expression by extra- or intracellular signals. First of all, the RNA polymerase themselves may be subjects of transcriptional regulation. Modifications of, e.g. the C-terminal domain (CTD) of the largest subunits of the Pol II multisubunit complex itself, may be directly involved in the interaction of Pol II with the promoter regions; each round of transcription is associated with reversible phosphorylation of the CTD (for review see Dahmus 1995). Post-translational phosphorylation and dephosphorylation of transcription factors is often necessary for their activation when a rapid response to external signals is required.

Two mechanisms have already been characterized for the action of protein kinases in the transmission of signals from the cell surface to the nucleus: activated protein kinases are translocated to the nucleus and phosphorylate their target transcription factors in the nucleus, realized, e.g., in the mitogen-activated protein (MAP) kinase cascades, or the transcription factors are stored in the cytoplasm and are translocated to the nucleus after phosphorylation, a process which was first demonstrated in animal cells for NF-kappa B (for review see Karin and Hunter 1995).

Another modification of proteins involved in regulational processes is the polyubiquitination which marks proteins for degradation by the 26S proteasome, e.g., the NF-kappa B inhibitor. If the inhibitor is subject to partial degradation by the ubiquitin system, NF-kappa B becomes active. Additionally, ligation of only a single or few ubiquitin molecules alter protein structures and functions and are probably involved in regulatory processes. The plant ubiquitin system is still poorly understood, but there is some evidence that the ubiquitin-dependent processes are closely associated with signal transduction pathways. It has already been shown that the ubiquitin system plays a role in plant pathogen interaction and stress response to abiotic stresses as well as in plant senescence probably for nitrogen recycling (for reviews see Belknap and Garbarino 1996; Van Kampen et al. 1996).

Furthermore, the chromatin structure has to be taken into consideration. Nucleosomes can inhibit the accessibility of the basal transcription initiation complex to promoter sequences as well as the binding of upstream regulatory proteins depending on their positioning.

Recently, a huge multisubunit complex, the SWI-SNF complex, of about half the size of a ribosome, has been identified that facilitates transcription by remodelling the chromatin under ATP hydrolyses (for review see Peterson and Tamkun 1995). The SWI-SNF complex can stimulate the binding of, e.g., yeast GAL4 transcription factor to a single binding site encompassed by a nucleosome by the factor of 10–30 *in vitro* (Côté et al. 1994). Not only the packaging of the DNA into nucleosomes but also a single component of the nucleosome, histone H1, can influence the DNA-binding activity of specific transcription factors (Schultz et al. 1996). Additionally, histone modifications, like, e.g., acetylation,

and DNA interaction with high mobility group (HMG) proteins of the 14/17 class have been observed to be associated with transcriptional active chromatin in animal cells. Furthermore, topological features of the DNA like supercoiling and/or DNA bending seem to influence transcription. Negative supercoiling enhances transcriptional initiation (Parvin and Sharp 1993) whereas positive supercoiling mediated by topoisomerases inhibits transcription (Gartenberg and Wang 1992), but the precise role of topoisomerases in transcriptional regulation is still unknown.

Gene copy number and DNA methylation also play their role in transcriptional regulation processes. Numerous examples now exist where the insertion of multiple copies of a transgene leads to loss of expression of some or all copies; even the expression of the endogenous gene can be influenced when the transgene contains homologous sequences (for review see Flavell 1994). Whether the multiple copies existing of numerous genes in most genomes influence the transcription of each other is not known yet. Often, cytosine methylation at 5'-CG-3' or 5'-CNG-3' residues is associated with gene repression, but the precise role of cytosine methylation in gene silencing has yet not been understood (Meyer and Saedler 1996).

Nevertheless, one of the most important steps in activating specific genes in response to external or internal signals is the interaction of specific *trans*-factors with their respective *cis*-elements in the promoter region of these genes. Therefore, in the last years many efforts concentrated on the isolation and characterization of the *trans*-factors and their respective *cis*-elements. Although the number of *trans*-acting proteins characterized for plant systems, especially for Pol II, has increased enormously in the last decade, our knowledge about the mechanisms of plant transcriptional regulation is still poor. Recently, it has been discovered that the same transcription factors can act as activators or repressors depending on their concentration and on the interacting partners (Shore 1994; Roberts and Green 1995). Furthermore, multiple *cis*-elements can be combined in one regulatory region.

For example, the G-box, a short sequence motif which interacts with specific proteins, appears often combined with other motifs rendering the promoter responsive to different external signals and stimuli. The G-box is involved in the responsiveness of plant promoters to light, anaerobiosis, p-coumaric acid and hormones such as ethylene, auxin, abscisic acid and methyl jasmonate. A large family of plant transcription factors, the G-box-like binding factors (GBF), interact with the G-box motif (for review see Menkens et al. 1995; see also Sect. 3.b). A second *cis*-element positioned nearby the G-box is required for responsiveness to different stimuli. The spacing between the two *cis*-elements seem to be critical, suggesting a direct interaction of the DNA-binding proteins with the GBF (for review see De Vetten and Ferl 1994; Menkens et al. 1995). These "building block" or modular systems enable the plant cell to react very precisely to different environmental or developmental signals with a small set of regulatory proteins and elements and to coordinate signalling via different pathways. Additionally, the formation of homo- and/or heterodimers between transcription factors increases the fine tuning of transcriptional regulation (for review see Brunelle and Chua 1993).

Pol I and Pol III have to deal with a quite different situation: hundreds to thousands of tandemly arranged gene copies are present in the genome. It is not yet clear whether all copies are used for gene expression or only a few are selected. How the active gene copies are selected, whether they are clustered or interspersed into silent copies, whether the transcription of one copy influences the transcription of other copies are all still unsolved questions. Loop structures identified microscopically favour a model that the spacer region between the transcription termination sites and the subsequent transcription initiation sites of the respective next repeating unit are brought in close contact and thereby recycle the Pol I complex (Sander et al. 1996).

As mentioned above, regulation and fine tuning of plant gene transcription are rather complex processes which are influenced by many parameters. We are far from understanding all single aspects and their integration in the mechanism of cell signalling. Therefore, we try to concentrate in this chapter on what is known about plant transcription factors and their *cis*-acting elements.

## 2. RNA Polymerase I

Pol I is exclusively active in a specific nuclear compartment, the nucleolus, transcribing the rRNA precursor (in plants approx. 32 to 35S in size) of three of the four ribosomal RNAs (18S, 5.8S and 25S rRNA). The genes of these rRNA species (rDNA) are located in high copy numbers on chromosomes ranging from one to several thousand in tandem arrays, representing the basis of the nucleolar-organizing region (NOR; Hadjiolov 1985). Here, the structural organization of the nucleolus is formed, where transcription of the rRNA genes, processing of rRNA and maturation of the ribosomal subunits occur (Motte et al. 1991). The correlation between size and number of NORs and nucleoli was firstly observed in the last century (Montgomery 1898); however, the tight association between rRNA genes and these organelles was clearly proved since the early 1960s (Ritossa and Spiegelman 1965).

The nucleolus shows three distinct structural components: (1) the fibrillar centre (FC), where the Pol I is localized (Scheer and Rose 1984) and where primary rRNA synthesis presumably takes place, although in very active plant nucleoli this component is less represented, and works on pea suggest that the boundary zone between FC and the dense fibrillar component (DFC) is the precise location of active Pol I (reviewed in Shaw and Jordan 1995); (2) the DFC, where several proteins, e.g. fibrillarin (Hugle et al. 1985), and small nuclear ribonucleoproteins (snRNPs; Kass et al. 1990), both associated with rRNA processing, are located, DFC has been proposed to be the site of early processing events of the rRNA precursor molecules; (3) the granular component (GC) composed by the ribosome precursor particles (rRNAs associated with ribosomal proteins) in later stages of maturation (Stahl 1982). These nucleolar components are variably represented in different eukaryotes as well as in different cellular stages.

The nucleolus undergoes disassembling and reassembling during the cell cycle; nevertheless, Pol I, topoisomerase and transcription factors, e.g., upstream binding factor (UBF), see Sect. 2.e), remain collocated with NORs in the condensed chromosomes during mitosis (Zatsepina et al. 1993; Gilbert et al. 1995). This stable affinity to its DNA template clearly distinguishes the Pol I transcription machinery from Pol II and III.

Most information on the Pol I transcription mechanism was derived from animal and yeast systems, so that references to these systems are often necessary. Nevertheless, improvement was achieved by working with plants and the most recent results underline similarities with animal systems but also peculiarities of the plant Pol I transcription machinery (for a review see Hemleben and Zentgraf 1994).

#### a) Structure of rDNA

In higher plants, rRNA genes are commonly found in tandem chromosomal arrays with the coding regions in the order 18S, 5.8S and 25S, following the direction of transcription. The rRNA-precursor in plants of 32S to 35S is generally smaller than that determined for animals (45S); nevertheless, the processing steps resulting in the mature 18S, 5.8S and 25S rRNA presumably occur in a similar manner (Rungger and Crippa 1977). Due to the essential role in ribosome structure and function, the rRNA coding regions are extremely conserved during evolution. However, high length and sequence heterogeneity of the rDNA repeats can be observed in higher eukaryotes from yeast (10 kbp) to mammals (44 kbp). In plants, this length heterogeneity is also evident ranging from 8 kbp (*Raphanus sativus*; Delseny et al. 1983) to 17 kbp (*Trillium, Paris*; Martini et al. 1982; Yakura et al. 1983). Length heterogeneity is also observed within the genome of an individual organism (Rogers and Bendich 1987; Hemleben et al. 1988). Minor differences already occur in the internal transcribed spacers (ITS1 and 2), but the greatest heterogeneity is located within the intergenic spacer (IGS; see Fig. 1). A more detailed analysis at the nucleotide sequence level reveals already reduced homology between plant species of the same genus ranging from 60 to 95% depending on the IGS region analysed, e.g. *Cucurbita maxima* and *C. pepo* (Kelly and Siegel 1989; King et al. 1993) or *Nicotiana tomentosiformis* and *N. sylvestris* (Volkov et al. 1996). Similarities become much lower in plant species of different genera of the same family, e.g. in Brassicaceae (Da Rocha and Bertrand 1996) or among *Lycopersicon esculentum* and *Solanum tuberosum* (Perry and Palukatis 1990; Borisjuk and Hemleben 1993) and are completely absent among representatives of different families (except for short motifs; Fan et al. 1995). This lack of evolutionary constraint was responsible for the wrong concept of IGS as "junk DNA" until the early 1980s, when it became clear that essential

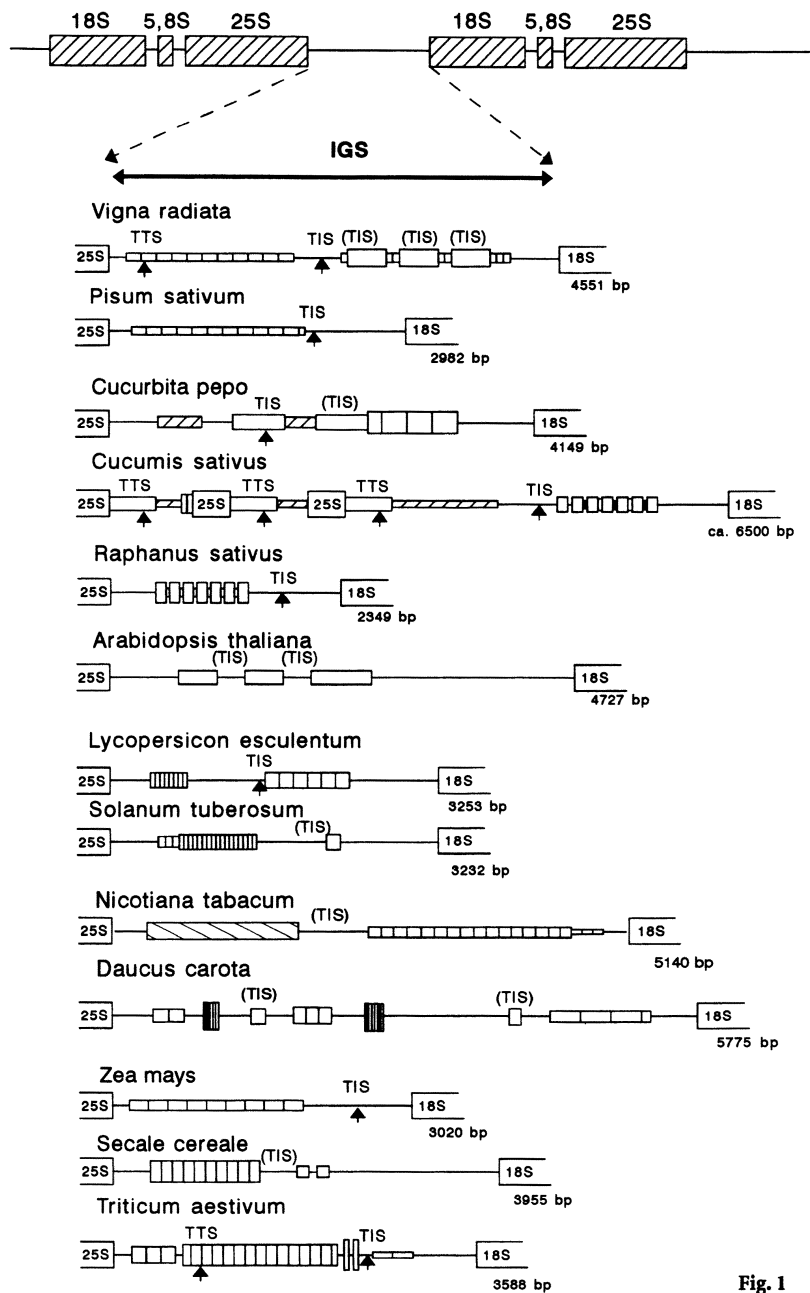


Fig. 1

sequences for rRNA transcription and its regulation as well as for rRNA processing and for rRNA replication are located within the IGS (for review see Moss and Stefanowsky 1995).

## b) RNA Polymerase I Promoters

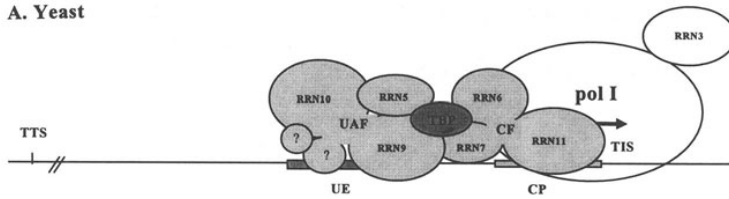
The first essential functional element that has been characterized in the IGS is the rRNA gene promoter containing the transcription initiation site (TIS, +1), mapped for several plants by *in vitro* transcription (*Vicia faba*, Kato et al. 1990; *Nicotiana tabacum*, Fan et al. 1995) or by primer extension or S1 mapping (*Brassica oleracea*, Bennet and Smith 1991; *Cucumis sativus*, Zentgraf et al. 1990; *Lycopersicon esculentum*, Perry and Palukatis 1990; *Pisum sativum*, Kato et al. 1990; *Raphanus sativus*, Delcasso-Tremousaygue et al. 1988; *Triticum aestivum*, Barker et al. 1988; *Vigna radiata*, Gerstner et al. 1988; *Zea mays*, Toloczyki and Feix 1986). The sequences directly surrounding the TIS are strictly conserved; therefore, now it can be detected by computer analysis (Fan et al. 1995). The consensus sequence TATA(G)TA(N)GGGGG, common to all Pol I promoters investigated, shows a highly conserved TIS containing a perfect TATA-box, which is absent in animals and yeast.

Essential *cis*-acting elements for the Pol I transcription system are quite well described for animal cells (Moss and Stefanowsky 1995), consisting of two essential and specifically spaced sequences, the core promoter (CP), approx. from nucleotide -45 to +15, and the upstream control element (UCE; or upstream binding element, UBE), approx. from nucleotide -160 to -110 (Fig. 2). Although these positions with respect to the TIS are highly conserved, a sequence comparison between different species did not allow the characterization of a consensus sequence for animals; nevertheless, between CP and UCE sequences within one promoter, e.g. CP and UCE in human, a certain degree of homology was found. Spacing of the CP and UCE appears crucial in rat, frog and yeast. Any insertion or deletion result in very deleterious effects on transcription and, as demonstrated by the "Xenopus paradox", spacing plays a role also in species-specificity: A 5-bp insertion between CP and UCE in *Xenopus* Pol I promoter produces a strong promoter in mouse cells while making this promoter non-functional in *Xenopus* cells (Culotta et al. 1987). Species

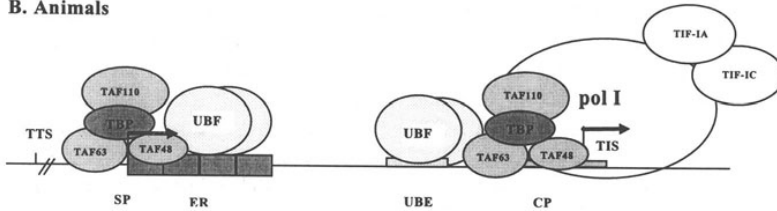
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Fig. 1. Intergenic spacer (IGS) region of a representative of rDNA repeats of several higher plants (modified after Hemleben and Zentgraf 1994). Transcription initiation site (TIS) and transcription termination site (TTS) are marked by an arrow if determined by S1 or mung bean nuclease or by primer extension mapping, or in parentheses if determined by computer search. Numbers of base pairs indicate size of IGS. References: *Vigna radiata* (Gerstner et al. 1988; Schiebel et al. 1989), *Pisum sativum* (Kato et al. 1990), *Cucurbita pepo* (King et al. 1993), *Cucumis sativus* (Ganal et al. 1988; Zentgraf et al. 1990), *Raphanus sativus* (Delcasso-Tremousaygue et al. 1988), *Arabidopsis thaliana* (Gruendler et al. 1991), *Lycopersicon esculentum* (Schmidt-Puchta et al. 1989; Perry and Palukatis 1990), *Solanum tuberosum* (Borisjuk and Hemleben 1993), *Nicotiana tabacum* (Borisjuk et al., in press), *Daucus carota* (Suzuki et al. 1996), *Zea mays* (Toloczyki and Feix 1986), *Secale cereale* (Appels et al. 1986), and *Triticum aestivum* (Barker et al. 1988; Vincenz and Flavell 1989)

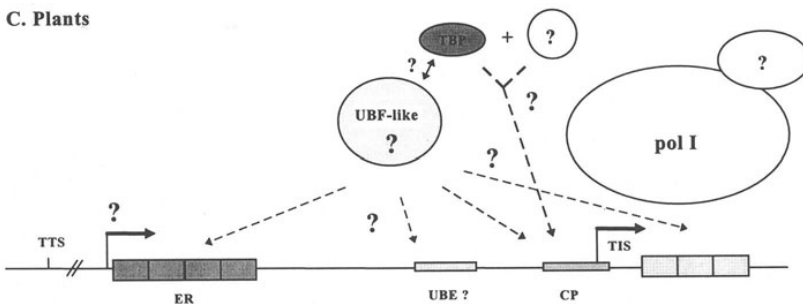
## A. Yeast



## B. Animals



## C. Plants



**Fig. 2.** Proposed models of Pol I transcription mechanisms in yeast (A modified after Steffan et al. 1996), animals (B modified after Goodrich and Tjian 1994) and plants (C combined data of several authors; see Table 1). Abbreviations: CF core factor; CP core promoter; ER enhancer repeats; *Pol I* RNA polymerase; RRN3 a putative yeast transcription initiation factor; RRN5, 9 and 10 UAF subunits; RRN6, 7 and 11 CF subunits, SP spacer promoter; TAF48, 63 and 110 TBP-associated factors; TBP TATA-box binding protein; TIF-IA and C transcription initiation factor; TIS transcription initiation site; TTS transcription termination site; UAF upstream activating factor; UBE upstream binding element; UBF upstream binding factor; UE upstream element

specificity is a well-known phenomenon in Pol I systems mainly studied in human, mouse and frog cells. Rapid IGS evolution allows clearly rapid compensatory mutations within the transcription machinery, selected and fixed within a population in a process called "molecular drive" (Dover 1992). Coevolution of the IGS and Pol I transcription machinery has resulted in a high degree of incompatibility between different organisms, e.g. mouse and human factors are functionally incompatible (Heix and Grummt 1995);

nevertheless, human and *Xenopus* enhancers are interchangeable, and even *Arabidopsis* enhancers are reported to function in *Xenopus* despite completely unrelated sequences (Doelling et al. 1993). These data, together with the "*Xenopus* paradox", led to the conclusion that in spite of a certain degree of species-specificity a common mechanism of rRNA transcription exists in eukaryotes.

A further interesting aspect concerning the organization of Pol I transcription in animal cells is the arrangements of "two transcription units" per tandem repeat (Reeder 1990). Initially observed in *Xenopus*, it was proposed also for mouse and *Drosophila* on the basis of structural similarities: One or more duplications of the *Xenopus* gene promoter, named spacer promoters, have been found to be transcribed until 60 bp upstream of the gene promoter; when spacer transcription occurs it silences other downstream spacer promoters. This observation supported the idea of a mechanism called "readthrough enhancement" that should keep the Pol I molecules tightly associated to the IGS until reaching the real TIS and thereby enhancing transcription (Mitchelson and Moss 1987).

This "two transcription units" organization as well as the presence of CP and UCE is controversially discussed for plants. Repeated elements are very common in plant IGSs (Fig. 1, Hemleben and Zentgraf 1994), but duplications are not restricted to the gene promoter (Gerstner et al. 1988; Kelly and Siegel 1989; Gruendler et al. 1991; King et al. 1993). Repeated elements containing the putative transcriptional termination site (TTS) have been found in *Vigna radiata* (Schiebel et al. 1989) and *Cucumis sativus* (Zentgraf et al. 1990); enhancer-like repeated elements are located upstream of the TIS in wheat (*Triticum aestivum*; Flavell et al. 1986), maize (*Zea mays*; Schmitz et al. 1989), potato (*Solanum tuberosum*; Borisjuk and Hemleben 1993) and probably occur in other plants. Finally, even part of the 25S rRNA coding region can be duplicated in *Cucumis sativus* (Ganal et al. 1988; Zentgraf et al. 1990). Based on these data the only common aspect appears to be the presence of repeated elements in the IGSs, which probably have a functional role in transcription, but a consistent "two transcription units" model with two or more active homologous promoters as found in animals is probably not realized in plants. On the other hand, strong indications for a promoter organization divided in CP and UCE have been found in *Cucumis sativus* (Zentgraf and Hemleben 1992) and *Triticum aestivum* (Ashapkin et al. 1995). However, functional promoter analysis in an *Arabidopsis* in vitro transcription system (Doelling and Pikaard 1995) led to different conclusions; in fact, these authors have found that the only essential element for Pol I activity is restricted to the surrounding sequences of the TIS (approx. -6 to +6). These divergent results need clearly further analysis and probably will be solved after isolation of the corresponding trans-acting-factors.



Noteworthy, the first result about species-specificity in plants was obtained using a tobacco *in vitro* transcription system (Fan et al. 1995). Tobacco (*Nicotiana tabacum*) and broad bean (*Vicia faba*) rDNA templates were tested in tobacco nuclear extracts, and primer extension experiments were performed: while the tobacco template was correctly transcribed and not affected by increasing amounts of  $\alpha$ -amanitin, no signal was present when broad bean template was used.

### c) Enhancer Elements

In spite of sequence heterogeneity, IGS of eukaryotes shows similar structural organization (Marilley and Pasero 1996), with the exception of *Saccharomyces cerevisiae*, so that the hypothesis of a functional role for the repetitive elements is largely accepted. In higher plants, evidences are given by *in vivo* and *in vitro* data. For hybrids between *Triticum/Aegilops* it was demonstrated that only the *Aegilops umbellulata* rDNA repeats, containing higher numbers of IGS repeated elements compared with *Triticum* rDNA, are transcriptionally active and form a nucleolus structure (Flavell et al. 1986). Similar effects have been observed in *Triticum/Secale* hybrids, where *Triticum* rDNA was predominantly transcribed (Capesius and Appels 1989). In mung bean (*Vigna radiata*; see Hemleben and Zentgraf 1994) deletions of IGS, tested in isolated nuclei, showed a consistent transcriptional activity only when several promoter-like repeats downstream of the TIS were present, whereas transcription products of shorter IGS deletions were almost undetectable.

Further support for a functional role of the IGS repeats was also given by *Arabidopsis* repeats cloned in *Xenopus* IGS which proved to stimulate transcription in *Xenopus* oocytes (Doelling et al. 1993). Recently, an exception to the listed data was observed by Doelling and Pikaard (1995), which revealed similar levels of transcription driven by the full length IGS containing the putative enhancer repeats and/or a minimal gene promoter. However, these results represent only first indications which, although relevant, must be further supported to propose a different mechanism of rDNA transcription between plants and animals. In animal cells, in fact, the essential role of enhancers is well known (Osheim et al. 1996), and it has been recently confirmed in detail in *Xenopus* (Mougey et al. 1996). Obviously, the enhancers of *Xenopus* appear to be associated with core histones, whatever the state of gene activity (Dimitrov et al. 1992), whereas normally enhancers of active genes are very accessible to micrococcal nuclease and those of inactive genes give a classic nucleosome ladder (Moss and Stefanowsky 1995). Because it is known that the upstream binding factor (UBF; see Sect. 2.e), that binds UCE and CP (Fig. 2) is able to bind enhancer repeats *in vitro* (Putnam and Pikaard 1992), it is commonly believed to be involved in ribosomal gene transcription-enhancing by repeat binding (Moss and Stefanowsky 1995).

#### d) Termination of Transcription

Surprisingly, sequences determining termination of transcription could be classified among sequences enhancing transcription in Pol I systems. In fact, although a conserved consensus sequence like the TIS cannot be identified, several experiments carried out with TTS, like the T3-box in *Xenopus* (Firek et al. 1989) or the Sal-box in mouse (Kuhn et al. 1988a), have suggested a role of TTS in transcriptional activation. The presence of inverted repeats like in the T3-box of *Xenopus* was found relatively well conserved also in *Vigna radiata* (Schiebel et al. 1989). Several distinct termination signals have been found also in *Cucumis sativus* which could be correlated with putative stem-loop structures probably having analogous functions of termination and enhancing of transcription (Zentgraf et al. 1990). Such inverted repeats, allowing the formation of complex stem-loop structures, bring the TTS in the proximity of TIS. This phenomenon could contribute to the already mentioned (Sect. 2.b) "read-through enhancement" of transcription by rapidly recycling the polymerase to the subsequent promoter (Schiebel et al. 1989).

#### e) Basal Transcription Mechanism and *trans*-Acting Factors

With the actual knowledge it is not possible to draw a complete scheme of Pol I transcription in plants; therefore, the models proposed for animals and yeast will be used to fill in the missing data for a putative plant model (Fig. 2). Although a comparison between *Xenopus*, *Drosophila* and mammalian systems is quite difficult, due to the still partial cloning of the genes coding for the numerous components of the transcription machinery, two basal factors, the TATA-box binding protein complex (TBP-complex) and the UBF, are well characterized. The TBP-complex (named SL1 in human, Comai et al. 1992, TIF-IB in mouse, Eberhard et al. 1993; factor D in rat, Tower et al. 1986; Rib 1 in *Xenopus*, McStay et al. 1991) is composed by the TBP plus three associated factors (TAFs), purified for human and mouse, 110/95, 63/68 and 48 kDa, in size, respectively (Comai et al. 1992; Rudolff et al. 1994). The TBP is probably not able to interact directly with DNA, but such interaction is realized by the 63/68 and the 48 kDa TAFs, the latter appearing to be responsible for the species-specificity (Beckmann et al. 1995). The low affinity to the Pol I promoter shown for this factor is mediated by UBF binding to CP and UCE, a protein which is essential for stabilization of the TBP-complex in *Xenopus* (Bodeker et al. 1996) and human (Bell et al. 1988), but surprisingly it is only acting as transcription-enhancer in mouse (Bell et al. 1990). UBF belongs to the high-mobility-group (HMG) proteins (Jantzen et al. 1990); it is quite well conserved from amphibians to mammals, contains four to five HMG-boxes, each one specialized for a different

function (Moss and Stefanowsky 1995), and binds DNA as homodimer in its phosphorylated form (Putnam et al. 1994; Voit et al. 1995). UBF of different organisms, e.g. human and frog, are interchangeable in sequence recognition in spite of complete lack of sequence homology (Moss and Stefanowsky 1995), showing high affinity for bent DNA (Marilley and Pasero 1996). DNA-binding of UBF represents the first step in promoter recognition so that it is commonly believed to be the gene activator (Fig. 2B); subsequently the resulting complex stimulates interaction of SL1 with the promoter, followed by the recruitment of Pol I and the initiation factors TIF-1A and TIF-1C (reviewed in Goodrich and Tjian 1994).

A similar transcription initiation complex can be described for yeast (Fig. 2A; Steffan et al. 1996). Here, two different protein complexes of three and five subunits, respectively, called core factors (CF) and upstream activator factor (UAF) bind to the CP and UCE [upstream element (UE) in yeast]. Interaction of UAF to the UE is necessary to commit the template to transcription. The essential transcription factor CF is then recruited to the template, following an interaction of TBP with UAF and CF. Interestingly, neither CF nor UAF complexes contain HMG-proteins. This stable pre-initiation complex is then able to recruit Pol I and the initiator factor RRN3 to the promoter. None of the components of the yeast transcription machinery shows any homology with factors of the animal system, with the exception of TBP (Steffan et al. 1996). Furthermore, in yeast, enhancer repeats are missing.

As it can be deduced from the data reported in Table 1, the situation in plants fundamentally resembles the model of animal systems, although showing some exceptions. The first reports of binding factors of maize described specific proteins which interact with the gene promoter as well as with repeated elements upstream of the TIS (Schmitz et al. 1989). Similar results have been obtained in wheat (Jackson and Flavell 1992) where the subrepeat A, upstream to the TIS, competed for the same factors which bind to the gene promoter supporting the putative enhancer function of these repeats (Flavell et al. 1986). In summary, DNA/protein interactions have been found (1) with spacer promoters (SP), enhancers and gene promoter in carrot (*Daucus carota*; Suzuki et al. 1996); (2) with enhancers and gene promoter in maize (Schmitz et al. 1989); (3) with UCE, CP and repeated elements in wheat (Jackson and Flavell 1992; Ashapkin et al. 1993, 1995) and cucumber (*Cucumis sativus*; Zentgraf and Hemleben 1992); (4) with gene promoter in broad bean (*Vicia faba*; Suzuki et al. 1995); and finally (5) factors interacting with AT-rich sequences have been found in *Arabidopsis thaliana* (Kneidl et al. 1995) and radish (*Raphanus sativus*; Echeverria et al. 1992, 1994; Echeverria and Lahmy 1995). Spacer promoters have been identified only in *Arabidopsis* and *Daucus*, and in the latter the spacer promoter competes for the same protein factor as the gene promoter.

Table 1. Proteins interacting with the intergenic spacer of rDNA in higher plants

Plant species	Protein or complex size	Binding site	Consensus sequence or position	References
<i>Daucus carota</i>	-	SP (CP)	(+1) TATATAGGGAGGGGG TATATAGGGAGGGGG	Suzuki et al. (1996) Suzuki et al. (1996)
<i>Zea mays</i>	27, 37, 38.5, 42, 44, 108 kDa	Repeated elements CP	Upstream TIS -40 to -9	Schmitz et al. (1989) Schmitz et al. (1989)
<i>Triticum aestivum</i>	- -	Repeated elements CP	Upstream TIS -34 to +10 (CATGG--GC-AAAAC)	Jackson and Flavell (1992) Jackson and Flavell (1992)
	65, 100, 240, 430, 970 kDa 100, 200, 400 kDa 90, 240, 420 kDa 45, 90, 240 kDa	Repeated elements UBE (?) CP (?) Downstream elements	Upstream TIS -270 to -126 -126 to -11 +191 to +366	Ashapkin et al. (1995) Ashapkin et al. (1995) Ashapkin et al. (1995) Ashapkin et al. (1995)
<i>Cucumis sativus</i>	16, 20, 24 kDa 16, 20, 24 kDa 16 kDa 70 kDa	UBE CP Repeated elements Repeated elements	-164 to -105 -42 to +16 Downstream TIS Downstream TIS	Zentgraf and Hemleben (1992) Zentgraf and Hemleben (1992) Zentgraf and Hemleben (1992) Zentgraf and Hemleben (1992)
<i>Vicia faba</i>	-	CP (?)	-94 to -34	Suzuki et al. (1995)
<i>Arabidopsis thaliana</i>	28.5 kDa	AT-rich region	-284 to -256	Kneidl et al. (1995)
<i>Raphanus sativus</i>	67 kDa	AT-rich region UBE/CP (?)	-1077 to -740 -120 to -55	Echeverria et al. (1992) Echeverria et al. (1992)

SP, spacer promoter; CP, cree promoter.

The size of the proteins or DNA/protein complexes, if known, are reported in Table 1. None of the genes coding for the mentioned proteins has been isolated yet, but *Cucumis* and *Arabidopsis* factors show analogies with HMG proteins in terms of affinity for bent DNA or single- and double-stranded DNA. Although uniformity of data is missing, a picture can be supposed as represented in Fig. 2C: A protein or a protein complex (UBF-like) with high affinity for bent DNA interacts with double-stranded DNA in a sequence-independent way, probably resolving the tight DNA/core-histone association; they contribute to the separation of the double-strand keeping the transcription fork open, allowing the Pol I recruited by a still unknown transcription factor(s) to contact DNA and initiate transcription. TBP, alone like in yeast (Steffan et al. 1996) or in a complex with TAF(s) like in animals (Beckmann et al. 1995), could play an important role in recruiting the Pol I to the template. It is now well known that TBP needs a perfect TATA-box to bind tightly to DNA (Juo et al. 1996), and a perfect TATA-motif is a specific feature of the plant Pol I promoter in comparison with animals and yeast. Furthermore, it was proven that TBP is able to interact with the maize IGS when supported by the yeast factor TFIIA (Haaß et al. 1994). It could be supposed then that TBP plus other still unknown essential factors recruit Pol I to the TIS. Finally, a "read-through enhancement" activity of the repeated elements could be proposed considering the affinity of these sequences to the factor(s) also binding the gene promoter as observed in carrot, cucumber, maize and wheat.

For Pol I in plants, it is clear that much more information is needed to complete our understanding of rRNA transcription and its coordinated regulation within the cell.

### 3. RNA Polymerase II

In contrast to Pol I, which is active in the nucleolus, Pol II transcription takes place in other compartments of the nucleus. Genes transcribed by Pol II are organized in chromatin loop domains anchored to the nuclear matrix by the matrix attachment regions (MARs) or scaffold attachment regions (SARs). The Pol II protein complex seems to be associated with the nuclear matrix and probably works hand in hand with topoisomerase II, one of the most prominent proteins located there. It is speculated that these loops domains are functional units of transcription; often enhancers are located close to the MAR elements.

### a) Basal Transcription Machinery

Whereas Pol I transcribes only a specific group of genes often occurring in high copy number, Pol II has to deal with numerous different genes so that the tight control over transcription initiation responding to very different developmental and environmental signals is performed by multiple activating or repressing factors interacting with the basal transcription factors (TFIIA, TFIIB, TFIID, TFIIIE and TFIIF). Pol II promoters can contain two different core elements acting independently or collectively: The TATA-box, typically located approximately 30 nucleotides upstream of the transcription initiation site, and the weakly conserved initiator which encompasses the initiation site. Most but not all class II promoters characterized so far contain either one or both of these core elements, but the sequences and spacing between them can vary significantly. The fact that the core promoters are extremely diverse makes it very difficult to predict general rules for the protein interactions occurring even with the basal transcription factors. Nevertheless, the first step in transcription initiation on the TATA-box containing promoters is the binding of TFIID to the TATA-box. TFIID is a large multiprotein complex. The TATA-binding activity is mediated by a 38-kDa subunit called TATA-binding protein (TBP). TBP has been characterized for numerous organisms including plants: The amino-terminal part of TBP is divergent and its function is not yet clear whereas the 180 carboxy-terminal amino acids consisting of two imperfect direct repeats show significant conservation (for review see Zawel and Reinberg 1995).

X-ray crystallography of yeast and *Arabidopsis* TBPs bound to the TATA-box motif revealed that the binding of protein induces a dramatic conformational change in the DNA. TBP binds in the minor groove of the DNA and leads to the severe bending of the DNA building the convex undersurface of the TBP saddle. This orientation exposes the entire upper surface of TBP for protein-protein interactions with a large number of diverse proteins reported to interact specially with TBP (for review see Burley 1996).

Mutations of TBP comprise transcription of all three RNA polymerases suggesting that TBP is involved in all cellular transcription processes and forms complexes with different TAFs (TBP-associated proteins) specific for the respective RNA polymerase (Cormack and Struhl 1992; Schultz et al. 1992). One or more TAFs are required for the response of Pol II to specific transcriptional activators (for review see Zawel and Reinberg 1995; Tjian 1996). In the absence of the TATA-motif, the TAFs can bind the initiator and surrounding sequences and thereby may anchor the TFIID complex to the promoter or initiator binding proteins (IBPs), e.g. YYI or TFII-I interact with TBP and/or other components of the transcription initiation complex. TATA-mediated and the initiator-mediated complex assembly pathways are not exclusive but may as well function synergistically. Using mutation analyses it seems that the initia-

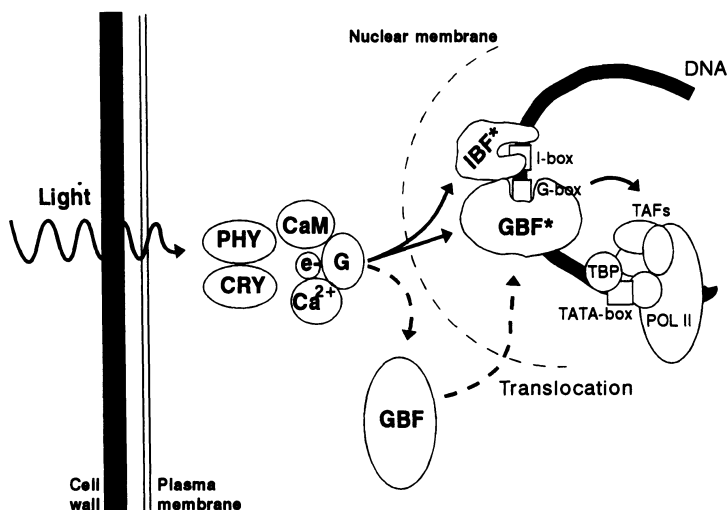
tor-mediated pathway is dominant *in vivo*, while *in vitro* the TATA-motif is the dominant element. Most of these approaches have been made in animal or yeast systems and only little is known about the plant basal transcription factors; however, it is likely that these are general features for eukaryotic cells and should also be true for plant cells. Nevertheless, it is not yet clear whether plant cells have adopted any specific mechanisms with respect to the basal transcription machinery.

#### b) *cis*-Acting Elements

The development of several convenient methods of molecular biology, such as promoter fusion to sensitive reporter genes in transgenic approaches and/or transient expression assays with electroporation or particle bombardment, have increased our knowledge of plant *cis*-elements dramatically in the last years. *Cis*-elements are defined as sequences which are relatively small, that confer different functions and expression patterns to a heterologous promoter and are very sensitive to specific mutations within the elements. In stress-inducible genes, a highly conserved 10-bp sequence (TCATCTTCTT) has been characterized interacting with a 40-kDa nuclear protein (TCA-1) of tobacco which is induced or modified by salicylic acid, a known inducer of pathogen related (PR) genes (Goldsbrough et al. 1993). In heat shock protein gene promoters, multimers of the heat shock element (NGAAN) are involved in the interaction with the trimeric heat shock factors (HSF; Schöffl et al. 1989; Lee et al. 1995; for review see Nover and Scharf 1997). Low temperature responsive genes share the short sequence motif A/GCCGAC which is thought to be the low temperature responsive element (LTRE; for review see Hughes and Dunn 1996).

An ubiquitous, *cis*-acting, regulatory DNA element found in many different plant promoters is the G-box, an hexameric motif (CACGTG). This sequence was first characterized in the 5' non-coding region of the light-regulated ribulose 1,5-biphosphate carboxylase small subunit gene (*rbcS*) and was thought to be unique to light-regulated genes (Giuliano et al. 1988).

Involvement of the G-box and G-box-like binding factors (GBF) in light-regulated gene expression is illustrated in Fig. 3. Further analyses now revealed that the G-box is essential for the response of many different promoters to a variety of environmental, developmental signals as well as to plant hormones like auxin, ethylene, abscisic acid (ABA) and methyl jasmonate. G-box elements can be grouped into two classes defined by the sequence flanking the hexameric core, especially by the nucleotides 3 or 4 bp up- and downstream of the core sequence. Two distinct types of GBFs, type A and type B, bind to class I and II G-boxes, respectively (Williams et al. 1992). According to this G-box classification, the *rbcS* G-box of *Arabidopsis* is a class I G-box bound by a type A GBF whereas *Antirrhinum* chalcone synthase G-box is a class II G-box bound by a type B GBF (Giuliano et al. 1988; Staiger et al. 1989).



**Fig. 3.** Role of GBF (G-box binding factor) and G-box in light-regulation of transcription. Signal perception seems to involve red and blue light receptors, phytochrom (*PHY*) and cryptochrom (*CRY*), which initiate, after activation by light, expression of light-responsive genes probably mediated by a G-protein (*G*),  $\text{Ca}^{2+}$ , Calmodulin (*CaM*), light-induced redox reaction ( $e^-$ ) and modulation of specific transcription factors (*IBF*; *GBF*). These factors interact with the light-responsive element consisting of the combination of an *I-box* and a *G-box*. Light-regulated translocation of *GBF* to the nucleus seems to be part of the activation process. Binding of *IBF* and *GBF* with respective *cis*-elements is followed by interaction with basal transcription factors (*TBP*, *TAFs*), and activation of RNA polymerase II (*Pol II*) to initiate transcription. (Modified after Menkens et al. 1995)

The G-boxes are located near the TATA-box (at approximately nt -50 to -500), and the DNA context and additional elements are critical for the appropriate response; e.g. the flanking *I-box* in the *Arabidopsis rbcS-1A* gene is necessary for its responsiveness to light (see Fig. 3), the EM2-box renders the wheat early methionine promoter sensitive to abscisic acid (Donald and Cashmore 1990; Guiltinan et al. 1990; for reviews see De Vetten and Ferl 1994; Menkens et al. 1995). Additionally, the spacing between the two *cis*-elements appears to be important (Block et al. 1990). Some G-box containing promoters are presented in Table 2.

More than one additional *cis*-element has been detected for the auxin responsive elements. For the *Glycine max* auxin responsive gene 28 (*GmAux28*) eight major protein-binding sites have been identified: two sequence motifs, TGACGACA (as-1/Hex related) and TCCACGTGTC (G-box), four AT-rich domains only modulating but no specifying auxin responsiveness, and two additional motifs, D1 (TAGTNNCTGT) and D4 (TAGTNCTGT), with a very similar core sequence also identified in several other auxin responsive elements (Nagoa et al. 1993). Two different



Table 2. G-box containing plant promoters responding to different environmental and hormonal stimuli. (After Menkens et al. 1995)

Stimulus	Organism	Regulated gene	Promoter region	G-box	Additional boxes
Auxin responsive	Soybean	GmAux 28		TCCACGTGTC	D1, D4, as-1/ Hex-related
Anaerobic responsive	<i>Arabidopsis</i>	Adh (alcohol dehydrogenase)	-281 to +1	TCGCACGTGFFGAC	CCCC-box
Abscissic acid responsive	Wheat	EM gene (early methionine)	-152 to -103	CGACACGTGGCG (EM1a) GCACACGTGCCG (EM1b)	EM2
Methyl-jasmonate responsive	Soybean	vspB gene (vegetative storage protein)	-585 to -535	TACACGTGCA	BoxI
Ethylene responsive	Tobacco	PR-1 gene (pathogen-related)	-213 to +1	TCTCACGTGATG	ERR
Light responsive	Parsley	CHS (chalcone synthase)	-175 to -124	TTCCACGTGGCC (BoxII)	BoxI
Light responsive	<i>Arabidopsis</i>	rbcS-1A	-320 to -125	TTCCACGTGGCA	I-box, L-box

G-box binding proteins, SGBF-1 and SGBF-2, have been isolated belonging to the basic region leucine zipper proteins (bZIP) both interacting with the same *cis*-element but exhibiting different functions in activating transcription (Hong et al. 1995). This indicates that the complex formations of the DNA-binding proteins with their *cis*-elements are even more complex than expected.

However, not all hormone responsive regions contain a G-box. Two short 7-bp motifs, TAACAAA and TATCCAC, play an important role in the gibberellic acid (GA)-regulated expression of the barley  $\alpha$ -amylase gene (Gubler and Jacobsen 1992). A conserved element between the promoters of wheat cathepsin B-like gene and  $\alpha$ -amylase gene of barley is required for full gibberellic acid response in both promoters indicating that gibberellic acid affects more than one mechanism of transcriptional control (Cejudo et al. 1992). Auxin responsive elements can be quite variable.

In *Pisum sativum*, an auxin responsive region (AuxRe) has been identified between -318 to -154 of the PS-IAA4-5 gene promoter. This auxin responsive region contains two domains: domain A (48 bp; -203 to -1569) with a T/GTCCCAT element acting as an auxin switch and domain B (44 bp; -299 to -256) containing aC/AACATGGN-C/A-A/GTGT-T/C-T/C-C/A element with enhancer-like activity. Both elements are highly conserved sequence motifs for various auxin-regulated genes, and DNase I footprinting revealed that both form DNA-protein complexes (Ballas et al. 1994, 1995). The soybean SAUR 15A (Small Auxin-Up RNA) gene contains a 30-bp sequence mediating most, if not all, auxin reactions. Additionally, two previously characterized elements, TGTCTC and GTCCCAT, have been identified within the more extended region necessary and sufficient for auxin induction (Li et al. 1994). In apices, the *parB* promoter contains two AuxRes, 48 bp (-210 to -163) and 95 bp (-374 to -280), responding independently to physiological auxin concentrations unlike the *as-1*/hex element/ASF-1 factor pathway responding only to higher concentrations of auxin (Takahashi et al. 1995). In the soybean GH3 D1 (25 bp) and D4 (32 bp) boxes, the TGTCTC elements are required but not sufficient for auxin induction, and additional upstream sequences are necessary which are responsible for constitutive expression when part or all of the TGTCTC elements have been mutated. In D1, the constitutive element overlaps with TGTCTC and binds specifically to a recombinant soybean protein with G-box specificity, whereas in D4, the constitutive element is separated from the TGTCTC motif (Ulmasov et al. 1996). An even higher complexity in combining *cis*-elements is realized, e.g. in the *cdc2a* promoter of *Arabidopsis*, one Myc, three Myb binding sites, one within an AuxRe, two AuxRe and one abscisic acid responsive element are present (Chung and Parish 1995).

AT-rich motifs are involved in gene expression of many different genes, e.g. the wound-responsive extensin gene of carrot, the tomato ethylene-responsive E4 and E8 genes, different embryo-specific expressed genes, like the lectin gene of soybean, the phytohaemagglutinin genes and the  $\beta$  phaseolin gene of French bean, or the sunflower helianthinin gene. AT-rich motifs are also thought to be the DNA counterpart in the HMG protein-DNA interaction (for review see Weising and Kahl 1992).

### c) *trans*-Acting Factors

One has to keep in mind that the assembly of the transcription initiation complex takes place in the context of the nucleus, where the DNA is packaged into chromatin, and thousands of different genes are competing for the pool of basal transcription factors. The formation of the initiation complex involves multiple steps: first of all, the DNA must be accessible for the transcription factors, specific transcription factors have to be translocated to the nucleus and/or have to be activated to contact their *cis*-elements on the DNA. Subsequently, the basal transcription factors assemble at the initiation site interacting with these specific transcription factors.

These regulatory proteins contain at least two functional domains: a sequence-specific DNA-binding domain and an activation (or repression domain) which interacts with the basal transcription machinery, most likely with TBF, TFIIB and TFIIF. Some activation domains have already been characterized in plant cells. Acidic domains are known from animal systems to have the potential to activate transcription. The acidic C-terminus of the maize C1 protein maize has been shown to function as an activation domain (Goff et al. 1991). Structural analyses revealed that an  $\alpha$ -helical stretch seems to be more important than the high negative charge of this region (Franken et al. 1994). Furthermore, in the HSF of tomato, the maize O2 and VP1 proteins, the rice OSH protein and the N-terminus of TGA1a of tobacco other acidic regions have been identified also acting as activation domains (McCarty et al. 1991; Treuter et al. 1993; Unger et al. 1993; Neuhaus et al. 1994; Tamaoki et al. 1995). Additionally, the acid activation domains of GAL4 of yeast and Vp16 of *Herpes simplex* function in plants, probably indicating a more general mechanism for transactivation by acidic domains (Ma et al. 1988; Goff et al. 1991). For GBF1 of *Arabidopsis* the activation domain is rich in proline residues (Schindler et al. 1992). This Pro-rich region seems to be conserved among some bZIP-proteins and, unlike the animal CTF Pro-rich activation domain, it is characterized by a large number of aromatic residues and only a few charged residues. In this case, a different mechanism appears to be realized in plants.

Plant transcription factors can be classified on the basis of their characteristic structural motifs (Table 3):

1. **bZIP proteins** (basic leucine zipper proteins). The bZIP motif comprises a basic amino acid stretch of about 25 residues containing the DNA as an  $\alpha$ -helical structure in the major groove adjacent to a leucine zipper where leucine residues are present every seventh residue over three to six repeat units. This leucine zipper is responsible for dimerization (Landschulz et al. 1988). All plant bZIP proteins characterized so far bind to DNA elements with an ACGT core sequence.

2. **bHLH proteins** (basic region helix-loop-helix proteins). Like the bZIP proteins, these proteins consist of a basic  $\alpha$ -helical region contacting the DNA and an HLH region composed of two  $\alpha$ -helices separated by a loop for dimerization. The bHLH proteins recognize a consensus sequence of CANNTG although the flanking sequences are also important for DNA-binding.
3. **Myeloblast-like proteins (MYB)**. The MYB domain is composed of three repeats consisting of 51–53 amino acids with the three tryptophan residues spaced at 18–19 amino acid intervals building up a hydrophobic core. The second and the third repeat are essential for DNA-binding; they contain three  $\alpha$ -helices, respectively, and the third helix in each recognizes the AACNG motif cooperatively. Most plant MYB proteins contain only the second and the third repeat with the exception of the potato Mybst1 with only one repeat in the middle of the molecule binding to GGATA-containing sequences (Baranowskij et al. 1994).
4. **HD proteins** (homeodomain proteins). The HD is composed of approximately 60 amino acids with three  $\alpha$ -helical stretches folded into a globular structure. Helices 2 and 3 form a helix-turn-helix motif at an angle of 120°, and helix 3 makes the contact with the major groove of the DNA. In some cases, e.g. the Knotted 1 product of maize, the HD is preceded by a 24-amino acid Glu-, Leu- and Lys-rich sequence called the ELK domain.
5. **MADS-box proteins**. The name is derived from the four initially identified members (MCM1 of yeast, AGAMOUS of *Arabidopsis*; DEFICIENS of *Antirrhinum*, SRF of human). The MADS-box is composed of 56 amino acids and can be divided into a basic and hydrophilic N-terminal region and a hydrophobic C-terminal region. The MADS-box proteins recognize the palindromic 10-bp consensus sequence CC(A/T)<sub>6</sub>GG.
6. **Zinc finger proteins**. The classic zinc finger is characterized by two Cys and two His residues that bind tetrahedrally to a zinc ion and several hydrophobic amino acids. Recently, a novel class of DNA-binding proteins has been characterized by the Dof domain containing a novel zinc finger motif which is larger than all known zinc fingers (Yanagisawa 1996).
7. **HMG-box proteins** (high mobility group-box proteins). The HMG-box is composed of about 80 amino acids and forms an L-shaped structure which interacts with the minor groove of the DNA, especially with irregular DNA structures such as four-way junctions. They have the capacity to bend the DNA and are therefore suggested to have an architectural role in assembly of higher order DNA/protein complexes (Grosschedl et al. 1994).
8. **Heat shock factors (HSFs)**. The N-terminal DNA-binding domain of these transcription factors is well conserved including a variant of the

**Table 3.** Some examples for each plant transcription factor class formed in the basis of their characteristic structural motifs, their binding motif and their biological function

Class	Name of transcription factor	Organism	DNA motif, which is bound by factor	Biological function
(1) bZIP Proteins				
	Opaque 2	Maize	Consensus: ACGT-core TCCACGTAGA, GGACACGTGTC ATGAGTCAT, GATGAPyPuTgPu, GACATGTC	Activates the promoter of the 22-kDa zein gene, of the b-32 gene (gene encoding an abundant albumin), of the 22kD-like alpha coixin gene, of the alpha prolamin gene
	GBF1	<i>Arabidopsis</i>	CCACGTGG (G-box), TGACGTGGT (hex motif)	Abscic acid-mediated gene activation
	EmBP1	Wheat	GCCACGTGGC	Root specific gene expression
	TAF-1	Tobacco	GCCACGTGGC, GTACGTGGCG (motif 1)	Gene expression during seed maturation
	CPRF-1/-3	Parsley	Box II and box III and other ATCG-core sequences	Light-regulated gene expression sequences(chalcone synthase promoter) Seed specific gene expression
	TGA 1a	Tobacco	TGACGT/C	TGA-1 highly expressed in roots, TGA-3 in all tissues
	PG 13	Tobacco		
	TGA-1/-3	<i>Arabidopsis</i>		
	OBF-4/-5	<i>Arabidopsis</i>		Bind to ocs elements which may function as oxidative stress responsive elements
(2) bHLH Proteins	RITA-1	Rice	C-boxes, G-boxes, A-boxes Consensus: CANN TG	Gene expression during seed development
	Lc (Leaf color)	Maize		Regulation of anthocyanin biosynthesis in combination with C1 (MYB- like protein)
	B-Peru	Maize		
	R-S	Maize		

(3) MYB-like proteins	Delia R	<i>Antirrhinum</i> Rice	Consensus: AACnG CGACTGCNGGTGC as B/C1 complex  CC(T/A)ACC  CCTACC TAAC(G/C)GTT (MBSI) TAACTAAC (MBSII)	] Regulation of anthocyanin biosynthesis  Regulation of anthocyanin biosynthesis (UPD-glucose flavonol 3-O-glycosyl transferase gene) Regulation of anthocyanin biosynthesis (NADPH-dependent reductase gene)  ] Phenylpropanoid biosynthesis  Trichome initiation process, anthocyanin biosynthesis Trichome initiation process Response to dehydration, salt stress, ABA cell shape  Leaf development, cell fate determination, mRNA trafficking through plasmodesmata Leaf morphology Leaf morphology, cell identity during early embryogenesis Leaf morphology
	C1 (Colorless 1)	Maize		
	P	Maize		
	PL	Maize		
	Zm-1/ -38	Maize		
	MBY305	<i>Antirrhinum</i>		
	MBY.Ph3	<i>Petunia</i>		
	TTG	<i>Arabidopsis</i>		
	GLABRA1	<i>Arabidopsis</i>		
	ATMYB2 Mixta	<i>Arabidopsis</i> <i>Antirrhinum</i>		
(4) HD proteins	Knotted1	Maize	TAACTG	
	KNAT1	<i>Arabidopsis</i>		
	OSH1	Rice		
	HvKnox3	Barley		

Table 3 (continued)

Class	Name of transcription factor	Organism	DNA motif, which is bound by factor	Biological function
(5) MADS-box proteins	<b>HD-Zip-group</b>			
	Athb-1	<i>Arabidopsis</i>	CAAT(A/T)ATTG	] Growth control
	Athb-2	<i>Arabidopsis</i>	CAAT(G/C)ATTG	
	HATs	<i>Arabidopsis</i>		
	<b>HD-PHD-finger</b>			
	GLABRA2	<i>Arabidopsis</i>		] Trichome and root development Signal transduction to COP1
	ATH1	<i>Arabidopsis</i>		
	<b>AG-group</b>		<b>Consensus: CC(A/T)<sub>0</sub>GG</b>	
	AGAMOUS	<i>Arabidopsis</i>		] Flower development
	PLENA	<i>Antirrhinum</i>		
	<b>AP3/PL-group</b>			
	DEFICIENS	<i>Antirrhinum</i>		] Stamen and petal development
	GLOBOSA	<i>Antirrhinum</i>		
	PISTILLATA	<i>Arabidopsis</i>		
	APETALA3	<i>Arabidopsis</i>		] Identity of floral meristem
	<b>API/AGL9-group</b>			
	APETALA1	<i>Arabidopsis</i>		] Expressed in vegetative tissues and in early floral meristems
	CAULIFLOWER	<i>Arabidopsis</i>		
	SQUAMOSA	<i>Antirrhinum</i>		
	<b>Others</b>			
	TM3	Tomato		





Table 3 (continued)

Class	Name of transcription factor	Organism	DNA motif, which is bound by factor	Biological function
(8) Heat shock factors	ATHSF HSF	<i>Arabidopsis</i> Tomato	Consensus: multiple (nGAAn)	Expression of heat shock proteins
(9) AP2/EREBP domain proteins	APETALA2	<i>Arabidopsis</i>		Floral organ specificity, meristem identity, seed coat development
	EREBPs	Tobacco	GCC-box	Bind to GCC-box of pathogenesis-related genes
(10) GT-1a/GT-2-type domain proteins	GT-1a	Tobacco	GTGTGGTTAAT	Binds box II element of light-responsive <i>rbc-S-3A</i> promoter
	GT-2	<i>Arabidopsis</i> , rice	GGTAAA/TT	Binds GT2- and GT3-box in <i>PHYA</i> promoter
(11) Proteins with other no obvious DNA-binding domains	TFHP-1	Tobacco	CACGTG	Activates horseradish peroxidase gene <i>prxC2</i>
	CG-1 BPF-1	Parsley Parsley	CGCG-containing sequences Box P	Box P of phenylalanine ammonia lyase promoter
	VPI	Maize	None	Involved in ABA-mediated gene regulation
	GF14	<i>Arabidopsis</i> , maize	None	Interacts with GBF factors

For references see Meshi and Iwabuchi (1995).

HTH (helix-turn-helix) motif and interacts with a multimer of a 5-bp module, the HSE (heat shock element, NGAAN). Downstream of the DNA-binding domain leucine zipper motifs are located responsible for, in this case, trimerization. The activation domain has been localized to the C-terminus (Rabindran et al. 1993; Treuter et al. 1993; Lee et al. 1995).

9. **AP2/EREBP domain proteins.** The AP2-domain, first characterized in APETALA2 of *Arabidopsis*, is a repeat unit of 68 amino acids. It shares considerable similarity with the 59-amino acids-long EREBP-domain characterized for a small group of tobacco proteins.
10. **GT-1a/GT-2-type domain proteins.** This DNA-binding domain has been characterized for the tobacco GT-1a protein which specifically binds to the box II light-responsive element (GTGTGGTTAAT). The DNA-binding domain of this small family of GT-like proteins can form four  $\alpha$ -helices and is immediately followed by a tetramerization domain.

**11. Proteins with other or no obvious DNA-binding domains.**

Some examples of the different classes of transcription factors mentioned above and their biological function are summarized in Table 3. An excellent review on plant transcription factors and their classification is given by Meshi and Iwabuchi (1995).

**d) Activation of *trans*-Acting Factors**

Activation of transcription factors by extracellular or intercellular signals can occur in different cellular compartments but then have to be translocated to the nucleus. In some pathways, the signalling molecule itself is translocated to the nucleus, while in others transcription factors are activated in the cytoplasm and thereafter transported to the nucleus. In animal and yeast systems, whole cascades of phosphorylation events have already been characterized to activate transcription factors, e.g. the mitogen-activated protein (MAP) kinase cascade (for review see Hill and Treisman 1995; Karin and Hunter 1995). Recently, several cDNA clones have been isolated from alfalfa, *Arabidopsis*, pea and tobacco with approximately 50% identity of the amino acid sequences to animal and yeast MAP kinases. The catalytic domains are highly conserved whereas the N-termini share little similarity indicating that different kinases recognize different substrates. The threonine and tyrosine residues where phosphorylation is required for the activity of the MAP kinase of yeast and animals are conserved in plants and seem to be crucial for the activity. Proteins with similarity to MAP kinase kinase (NPK2) and MAP kinase kinase kinase (CTR1/NPK1) have been isolated from tobacco demonstrating that the MAP kinase cascade might be a conserved signalling pathway in all eukaryotic cells (Banno et al. 1993; Kieber et al.

1993). A comparison of vertebrate and plant MAP kinase cascades is illustrated in Fig. 4. In plants, the MAP kinase cascade seems to be involved in ethylene signalling and in auxin-induced cell proliferation. Whether plant MAP kinases are also involved in other signal transduction pathways, e.g. osmoregulation as it is realized in yeast, has to be elucidated (for review see Jonak et al. 1994).

Circadian rhythms (for review see Piechulla 1993; Anderson and Kay 1996) can also be generated by phosphorylation events, e.g. the periodic activity change of the phosphoenolpyruvate carboxylase (PEPc) of *Bryophyllum* (Kalanchoe) is due to periodic phosphorylation which is achieved by the periodic synthesis and breakdown of a serine kinase, the PEPc kinase (Wilkins 1992). Another class of serine/threonine kinases characterized in plants is located in the plasma membrane, and the members are called the receptor-like protein kinases (RLKs) showing structural similarities to the polypeptide growth factor receptor of animals. The RLKs can be classified into three groups based on structural similarities in their extracellular domain: First, the S-domain class, re-

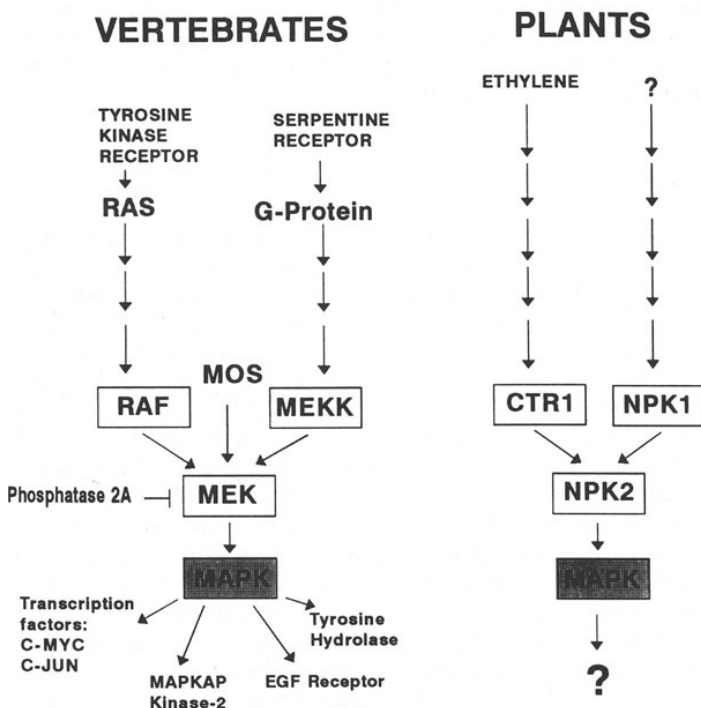


Fig. 4. Summary of MAP kinase signal transduction pathway comparing vertebrates and plants. MAPK MAP kinase; MEK and NPK2 MAP kinase kinase; RAF, MEKK, CTR1 and NPK1 MAP kinase kinase kinase. (Modified after Jonak et al. 1994)

lated to the self-incompatibility locus glycoproteins (SLG) of *Brassica*; second, the leucine-rich repeat class containing a tandemly repeated motif found in numerous proteins of many eukaryotes; and the third class showing epidermal growth factor-like repeats. The diversity among plant RLKs reflects their far-reaching implication for signal perception and signal transduction in plants (for review see Walker 1994).

#### 4. RNA Polymerase III

Pol III has an even more difficult job to do: It has to deal with numerous different genes with at least three different classes of promoters. One class of Pol III promoters is predominantly formed by the tRNA promoters consisting of two variably spaced 10-bp elements, box A and box B. In striking contrast to Pol I and II promoter elements, box A and box B are located within the coding region of the genes downstream of the transcription initiation site. Whereas the distal box B contains the high affinity binding site for TFIIC, box A orients TFIIC in the 5' end of the coding region and sets the initiation site approximately 20 bp upstream of box A. TFIIC initiates the formation of a Pol III complex (for review see Zawel and Reinberg 1995). The second class of Pol III promoters is exemplified by the 5S rRNA genes. The 5S rDNA promoter contains another intragenic control region (box C) interacting with TFIIIA, a polypeptide containing multiple zinc finger domains, which subsequently associates with TFIIC to initiate Pol III complex formation (for review see Zawel and Reinberg 1995). The third class of Pol III promoters is represented by the U6-snRNA promoter, paradoxically containing typical Pol II promoter elements. The human U6-snRNA genes lack internal control elements but contain a TATA-motif at approximately -30 and proximal sequence element (PSE) typical for Pol II-transcribed snRNA genes. However, Pol III specificity results from the combination of the TATA-motif with the PSE which interacts with the Pol II complex SNAPc. Nevertheless, exact spacing of the TATA-motif and the PSE/SNAPc complex results in a surface for Pol III. In contrast, yeast U6-sn RNA promoter contains an intragenic A and B box and a TATA-motif, but no PSE is present. Here, the TATA-motif is not essential for transcription, the initiation of the Pol III complex formation is also mediated by binding of TFIIC. Subsequently, in all types of Pol III promoters the multisubunit factor complex TFIIB, which includes TBP, joins the initiation complex and mediates the entry of the Pol III (for review see Zawel and Reinberg 1995). As already described (Sects. 1 and 2.d) for Pol I, yeast Pol III transcription complexes are recycled *in vitro*; a model proposes the direct transfer from the termination site to the promoter (Dieci and Sentenac 1996). An additional factor TFIIE, essential for transcription of tRNA and 5S rRNA genes, seems to be involved in the

reutilization of transcriptional complexes, but its action is not restricted to reinitiation events (Dieci et al. 1993).

Plant Pol III transcribed genes are poorly investigated. The basal components of the transcription machinery seem to be conserved, but there are also some plant-specific features. In contrast to yeast and animals, where only a single gene encodes TBP, two TBP isoforms have been isolated from *Arabidopsis* and maize which can substitute for yeast and human TBP in vitro and in yeast mutants (Mukumoto et al. 1993; Vogel et al. 1993). The two *tbp* genes in maize are differentially expressed in different tissues indicating non-overlapping functions in plants (Vogel et al. 1993). The promoter elements of two different 7SL genes of *Arabidopsis* are identical to those of the U-snRNA genes indicating that some plant genes have changed the Pol III promoter class during evolution (Heard et al. 1993). Additionally, there seem to be differences in the promoter elements of the snRNA genes between monocot and dicot plants (Connolly et al. 1994). A TFIIC homologue has already been isolated from *Dictyostelium discoideum* which can functionally substitute human TFIIC. This 380-kDa multicomponent factor also binds to the B-box element of the tRNA promoters without significant A-box interaction (Bukenberg et al. 1994).

## 5. Concluding Remarks

Despite the enormous amount of new information which has accumulated in the last decade concerning transcriptional regulation in plants, our understanding of the answering of plant cells to various environmental or endogenously produced developmental stimuli is still poor. Signal perception as well as signal transmission to the nucleus are still subject of intensive investigations in the field of plant science. Many gaps in our knowledge, especially for Pol I and Pol III transcriptional regulation, need to be filled for plants. Obviously, the coordination of transcriptional regulation of the three different systems working simultaneously in the nucleus will be an interesting topic for future investigations. TBP, which is involved in all transcriptional initiation events (for review see Zawel and Reinberg 1995), seems to be one good candidate for a coordinated transcriptional control of the three systems. In contrast to yeast and animal cells, two TBP isoforms have already been isolated from *Arabidopsis* and maize; the two maize *tbp* genes are differentially expressed in different tissues indicating that in plants slightly different transcription mechanisms occur. Furthermore, a cross-talk between nucleus and chloroplast is realized by a feedback mechanism that conditions nuclear gene expression suggesting a complex network of interactive signalling components acting in plant cells (von Arnim and Deng 1996).

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## Genetics of Plant Pathogenic Fungi

By Paul Tudzynski and Bettina Tudzynski

### 1. Introduction

Plant pathogenic fungi still represent a severe threat to the agricultural industry, especially since the chemical control of these organisms has become more complex. Due to environmental protection legislation, the costs for tests of new fungicides have been multiplied – whereas fungi rapidly develop resistance against the established chemicals. As a consequence, there is an increasing pressure on the development of alternative plant protection systems. On the pathogen side, "Achilles' heels", i.e. new targets for specific fungicides, have to be defined; this gives new input into intensive basic research on potential pathogenicity factors (and, of course, defence mechanisms on the plant side). On the other hand, results obtained in the last years in this area have shown that the study of host-pathogen interaction gives invaluable insights to general cell biological phenomena in both partners, including cell-cell interaction, signal perception and transduction, gene activation, etc.. Therefore, basic research in this field has been considerably intensified, as much in academics as in commercial laboratories.

This chapter is based on the last article of this series (Tudzynski and Tudzynski 1996). Due to the increasing number of research groups in the field and accordingly a large number of relevant publications, this chapter focuses on some trends and will just present examples, not complete compilations of interesting systems and results. In particular, the area of molecular analysis of genetic complexity of pathogenic fungi has become very broad: therefore the authors have not tried to update the compilation given in Tudzynski and Tudzynski (1996). Polymerase chain reaction (PCR)-based techniques are now widely used in systematics and population genetics for the characterization of subgroups and races of fungi (e.g. Overmeyer et al. 1996), and for the detection and quantification of pathogenic fungi in infected tissue (e.g. Mahuku et al. 1995; Achenbach et al. 1996; Schilling et al. 1996). Restriction fragment length polymorphisms (RFLP) and random amplified polymorphic DNAs (RAPD) have been used in a variety of fungi for genetic analysis and gene mapping (e.g. in *Phytophthora sojae*, Tyler et al. 1995; in *Mag-*

*naporthe grisea*, Zhu and Dean 1996). A new technique originally developed for genetic mapping in plants, amplified fragment length polymorphisms (AFLP; Vos et al. 1995), a kind of "hybrid" between RFLP and RAPD, based on the selective PCR amplification of restriction fragments from digests of genomic DNA, has been successfully applied to fungal systems (e.g. Majer et al. 1996) and is receiving more and more interest, since it yields more reliable data than RAPD. This short chapter will focus on the progress made in the analysis of basic compatibility factors, on a set of methods designed to identify "new" genes involved in the interaction, and on the role of signal chains in the pathogen.

Several aspects of the genetics of phytopathogenic fungi and the interaction with their hosts have been covered by recent monographs and conference proceedings (e.g. Honée and Nürnberger 1995; Kohmoto et al. 1995; Stacey et al. 1996; Carroll and Tudzynski 1997), general reviews on host-parasite interaction (e.g. Oliver and Osbourn 1995; Hensel and Holden 1996; Jackson and Taylor 1996; Knogge 1996; Leong 1996) and detailed reviews on specific topics, e.g. plant cell wall degradation and penetration by pathogenic fungi (Walton 1994; Howard and Valent 1996; Mendgen et al. 1996), genetics of specific fungi or systematic groups (e.g. Banuett 1995; Judelson 1996), elicitors (Hahn 1996), toxins (Walton 1996), and degradation of plant defence compounds (VanEtten et al. 1995; Osbourn 1996). Several excellent reviews and books on plant resistance and defence reactions appeared in the last years, e.g. Kombrink and Somssich 1995; Ebel and Scheel 1997; Heitefuss et al. 1997).

## 2. Genetic Analysis of Compatibility Factors: An Update

As outlined in the previous review (Tudzynski and Tudzynski 1996), there are basically two genetic systems influencing the interaction of phytopathogenic fungi and their hosts: the so-called pathogenicity or compatibility factors, the "weapons" which allow penetration and colonization of the host in spite of the plant's basic defence systems, and the so-called incompatibility factors involved in specific gene-for-gene systems [this highly interesting area has been reviewed by Knogge (1996) and therefore is not treated here]. The bulk of research activities in the last years has been devoted to the analysis of compatibility factors. Molecular genetic methods allow functional analyses of single factors by isolation of specific genes, studying their expression in planta and characterizing the effect of deletion mutants. Many genes have been cloned since the last review of this series, either by specific approaches (heterologous probes, PCR-based techniques, etc.) or "black box" approaches (differential cDNA techniques, insertional mutagenesis; see Sect. 3), and several have been knocked out by targeted gene disruption/gene replacement (see the examples listed in Table 1). However, since the "early" disruption studies had yielded more or less disappointing results, a new trend is recognizable; more and more research groups have started to follow-up expression of genes in planta, e.g. by reverse-

Table 1. Examples of functional analysis of potential fungal compatibility genes by gene disruption and expression studies. (Updated from Tudzynski and Tudzynski 1996)

Factor	Fungus	Gene(s)	Expression in planta	Phenotype of deletion mutant(s)	References
<b>Cell wall degrading enzymes</b>					
Cutinase	<i>Nectria haematococca</i>	<i>cut1</i>	+	No	Stahl and Schäfer (1992)
		<i>cut1</i>	+	Decrease in virulence on pea	Rogers et al. (1994)
	<i>Magnaporthe grisea</i> <i>Alternaria brassicicola</i> <i>Botrytis cinerea</i>	<i>cut1</i>	+	No	Stahl et al. (1994)
		<i>cutab1</i>	+	No	Sweigard et al. (1992)
		<i>cut1</i>	+	No	Köller et al. (1995) van Kan et al. (1997)
Cellulase	<i>Cochliobolus carbonum</i>	<i>cel1</i>	?	No	Sposato et al. (1995)
	<i>Claviceps purpurea</i>	<i>cel1</i>	+(RT-PCR)	No	Müller and Tudzynski (1996), Müller et al. (1997)
Xylanase	<i>C. carbonum</i>	<i>xyl1</i> -3	+(xyl1 + 3)	No	Apel et al. (1993), Apel-Birkhold and Walton (1996)
		<i>xyl1</i> ( <i>xyn22</i> ) <i>xyl2</i>		even $\Delta$ -xyl1,2,3)	Wu et al. (1995) Wu et al. (1996)
	<i>M. grisea</i>	<i>xyl1</i> ( <i>xyn33</i> ) <i>xyl2</i>		No (even $\Delta$ xyl1,2)	
Pectate lyase	<i>C. purpurea</i>	<i>xyl1</i>	+(RT-PCR)	No	Giesbert and Tudzynski (1996 and unpubl.)
		<i>xyl2</i>	+(RT-PCR)	NT	
		<i>pelA</i>	+(RT-PCR)	No	Guo et al. (1995a,b)
		<i>pelB</i> <i>pelC</i>	+	NT NT	
Pectin lyase	<i>Glomerella cingulata</i>	<i>pl1</i>		No	Bowen et al. (1995)



Table 1 (continued)

Factor	Fungus	Gene(s)	Expression in planta	Phenotype of deletion mutant(s)	References
Polygalacturonase	<i>C. carbonum</i>	<i>pgn1</i>		No	Walton and Scott-Craig (1996)
		<i>pgx1</i>		( $\Delta$ pgn1/pgx1)	
	<i>Penicillium olsonii</i>	<i>pg1</i>		No	Kusserow and Schäfer (1994)
	<i>Cryphonectria parasitica</i>	<i>enpg1</i>	+	No	Gao et al. (1996)
	<i>B. cinerea</i>	<i>bcpga1</i>	+		ten Have et al. (1996)
Proteases	<i>C. carbonum</i>	<i>alp1</i>		No	Murphy and Walton (1996)
	<i>C. parasitica</i>	<i>eapc</i>		No	Jara et al. (1996)
Detoxification of plant defence agents					
Cyanide hydratase	<i>N. haematococca</i>	<i>cht</i>	+	No	VanEtten et al. (1994)
Pisatin demethylase	<i>N. haematococca</i>	<i>pda</i>	+	No(?)	VanEtten et al. (1994)
Maackia detoxification	<i>N. haematococca</i>	<i>mak1</i>	+	Reduced virulence ( <i>Cicer arietinum</i> )	Covert et al. (1996) Enkerli et al. (1996)
Avenacinase	<i>Gaeumannomyces graminis</i>		+	Altered host specificity (no pathogenicity on <i>Avena</i> )	Bowyer et al. (1995)
Avenacinase-like enzyme	<i>B. cinerea</i>	<i>sap1</i>	?	? (No influence on tomato and bean)	Quidde and Tudzynski (1996)
Toxins					
T-toxin	<i>C. heterostrophus</i>	<i>pks1</i>	+	Reduced virulence on T maize	Yang et al. (1996)
HC-toxin	<i>C. carbonum</i>	<i>his1</i>	+	Reduced virulence on toxin-sensitive maize varieties	Panaccione et al. (1992)

Tricothecenes	<i>Gibberella pulicaris</i> <i>G. zeae</i>	<i>tox5</i> <i>tri5</i>	?	Reduced virulence on parsnip Reduced virulence on wheat and oat	Desjardins et al. (1992) Proctor et al. (1995)
Enniatin	<i>Fusarium avenaceum</i>	<i>esyn1</i>	?	Reduced pathogenicity on potato tuber tissue	Herrmann et al. (1996)
Cercosporin	<i>Cercospora kikuchii</i>	<i>le6</i>	?	Reduced virulence on soy bean	Upchurch (1995)
<b>Cell wall associated proteins</b>					
Hydrophobin	<i>M. grisea</i>	<i>mpg1</i>	+	Reduced formation of appressoria	Talbot et al. (1993)
Cerato-ulmin	<i>Ophiostoma novo-ulmi</i>	<i>cu</i>	?	No	Bowden et al. (1996)
Repellent	<i>Ustilago maydis</i>	<i>rep1</i>	?	? (Reduced aerial hyphae and surface hydrophobicity)	Wösten et al. (1996)
<b>Miscellaneous</b>					
Pheromone response factor	<i>U. maydis</i>	<i>prf1</i>	?	Loss of pathogenicity	Hartmann et al. (1996)
Superoxide dismutase	<i>Glomerella graminicola</i>	<i>sod1</i>	?	Reduced virulence	Fang and Hanau (1996)
G protein ( $\alpha$ -subunit)	<i>C. parasitica</i>	<i>cpg1</i>	+	Loss of virulence	Gao and Nuss (1996)
		<i>cpg2</i>		+	No
Melanin biosynthesis	<i>Colletotrichum lagenarium</i>	<i>thr1</i>	+	Reduced virulence	Perpetua et al. (1996)
Nitrogen regulation	<i>M. grisea</i>	<i>nut1</i>	?	No	Froeliger and Carpenter (1996)
NT, not tested.					

transcription-PCR (RT-PCR) or by reporter systems like glucuronidase (GUS) or more recently green fluorescent protein (GFP), (e.g. Spellig et al. 1996) and "tagged" proteins (which allow immunological detection of gene products). Deletion mutants are not just checked for the ability to induce disease symptoms, but are characterized in more detail in planta, i.e. the importance of cytological/structural analysis is being recognized.

The role of **cell wall degrading enzymes** (CWDE) in pathogen-plant interactions has been investigated in a wide variety of systems; many genes have been cloned and some of them knocked out (see Table 1), but to what degree those enzymes really contribute to a successful penetration and colonization of host tissue or whether most of them just have nutritional tasks is still open to question (see recent reviews by Howard and Valent 1996; and Mendgen et al. 1996). This holds true for the role of cutinases. At least for fungi which produce infection structures like appressoria the osmotic pressure reached in these structures (dependent, e.g., on melanin biosynthesis, see Chumley and Valent 1990) is more important than the enzymatic activity on cutin. Köller et al. (1995) suggested that in *Alternaria brassicicola* the main cutinase activity in axenic culture (which can be knocked out without any effect on pathogenicity) has mainly nutritional importance, whereas two other cutinolytic activities present mainly in planta are responsible for pathogenicity.

For the other CWDEs the situation turned out to be rather complex: (1) most enzymatic activities of a particular class are redundant; e.g. in the best investigated system, *Cochliobolus carbonum* (Walton and Scott-Craig 1996), there are at least two polygalacturonases (PG) encoded by two genes, three  $\beta$ -1,3 or  $\beta$ -1,6-glucanases (encoded by two genes), three proteases (two genes), and at least five xylanases (probably four genes); (2) the various enzymes of a class differ in their substrate specificity (endo-, exo-) and in the regulation of their encoding genes; e.g. *xyl1* of *C. carbonum* is expressed in planta and in vitro, *xyl2* only in vitro, *xyl3* only in planta (Apel-Birkhold and Walton 1996). In *Cryphonectria parasitica* the product of *enpg1* is responsible for the main PG activity in vitro, but in planta it shows minor activity, whereas two other PGs are present in planta only (Gao et al. 1996). In *Botrytis cinerea* there are at least five PG genes, one of which is constitutively expressed, the others are inducible, possibly by the products of the first one. The constitutive PG gene recently has been knocked out; the influence on pathogenicity is not yet clear, however (ten Have et al. 1996); (3) knocking out the gene for one enzyme of a specific class is not expected to have a dramatic effect, and even double and triple knock outs had no effect on pathogenicity (see Table 1); in some cases it could be shown that knocking out one or several prominent enzyme activities leads to the activation/expression of "silent" genes coding for enzymes which can compensate the loss, e.g. additional xylanases appear in a *xyl1/xyl2* double mutant of *M. grisea* (Wu et al. 1996). So far there is only one example where

deficiency of an enzymatic activity results in decreased pathogenicity: a non-pathogenic mutant of *Colletotrichum magna* has been shown to be deficient in pectate lyase secretion (not in synthesis) (Wattad et al. 1995).

The authors have started a concerted approach to study the role of cell wall degrading enzymes in the interaction of *Claviceps purpurea* and rye by analysing the degradation of the different cell wall components and expression of the corresponding genes/presence of enzymes in situ by microscopic and molecular methods (immunogold/enzyme gold labelling, in situ hybridization, RT-PCR, etc.), using also disruption mutants (Tenberge et al. 1996; Müller et al. 1997). This approach will allow the authors to determine the relative importance of degradation of the different cell wall components (is removal of either of them enough, is one component essential, etc.), and can prove if a knock out mutant really is no longer able to degrade a specific substrate.

**Detoxification of host defence compounds**, either inducible (phytoalexins) or preformed (phytoanticipins), has been studied in several fungi (see recent reviews by VanEtten et al. 1995; Osbourn 1996). Though disruption of the pisatin demethylase (*pda*) gene of *Nectria haematococca* had indicated that this gene most probably is not essential for pathogenicity on pea, the expression of this gene has been studied in detail; it is induced in a highly specific way by pisatin, and the corresponding pisatin-specific transcription factor has been characterized (He et al. 1996). The riddle of the close linkage between *pda* activity and pathogenicity of *N. haematococca* on pea (which had led to the idea that *pda* itself is essential) has been solved: Liu et al. (1996) could prove that there are at least two pea pathogenicity (*pep*) genes, closely linked to *pda* on a dispensable chromosome, the exact function of which is still open to question. Li et al. (1995) cloned and characterized a gene from *Fusarium solani* f. sp. *phaseoli*, encoding kievitone hydratase, which detoxifies the bean phytoalexin kievitone. Another gene involved in phytoalexin degradation, *mak1*, the product of which converts maackianin (from *Cicer arietinum*) to less toxic compounds, has also been found to be localized on a minichromosome in *N. haematococca* (Covert et al. 1996). In contrast to *pda*, its disruption seems to have effect on virulence of the fungus on chickpea (Enkerli et al. 1996). Therefore, the discussion on the vital importance of phytoalexin degradation is not yet closed.

The detoxification of the *Avena* root saponin, avenacin, by *Gaeumannomyces graminis* isolates had been one of the first examples of an essential factor determining host specificity of the fungus (Bowyer et al. 1995). Avenacinase activity and a putative avenacinase gene have been detected also in several isolates of *Fusarium avenaceum*, indicating that this activity is of general importance for *Avena* pathogens (B. Tudzynski and A. Jülich, unpubl.). *Septoria avenae*, a leaf pathogen, degrades the oat leaf saponins, avenacosides. A corresponding gene has been cloned

(Wubben et al. 1996). In *Septoria lycopersici*, a highly homologous gene has been cloned coding for a tomatinase, an enzyme detoxifying the tomato saponin tomatin by removing a terminal glucose (Osbourn et al. 1995; Sandrock et al. 1995), gene disruption so far has not been achieved in this system. Tomatin-degrading activities have been described and studied in detail in several other pathogens of tomato, which use different detoxification mechanisms, e.g., various *Fusaria* and *B. cinerea* (see Osbourn 1996; Quidde and Tudzynski 1996; Wessels and Weltring 1996).

*Botrytis cinerea* has been shown to detoxify tomatin by removal of a terminal xylose, in contrast to all other fungal systems described so far. A field isolate from *Vitis vinifera* lacking tomatinase activity showed significantly increased sensitivity to tomatin and strictly reduced pathogenicity on tomato, but normal pathogenicity on bean (Quidde and Tudzynski 1996). A gene (*sap1*) has been cloned using the tomatinase gene of *S. lycopersici* as a probe. Its predicted product reveals high homology to the avenacinase of *G. graminis* and the tomatinase of *S. lycopersici*. Targeted disruption of *sap1*, however, did not abolish tomatinase activity but a highly specific glucosidase activity against avenacin. The relevance of this result has to be investigated, since *B. cinerea* is no pathogen of *Avena*. The deletion mutants are being tested on a variety of host plants of *B. cinerea* (Quidde and Tudzynski, unpubl.). However, these data show that *B. cinerea* has at least two different saponin-specific glycosidases, a tomatin-specific xylosidase (the corresponding gene is being cloned by a reverse genetic approach now) and an avenacin-specific glucosidase.

Obviously detoxification of plant saponins is a widespread and important capability of phytopathogenic fungi, and it might turn out to influence or even determine host specificity in several systems.

Phytotoxins are synthesized by a large number of phytopathogenic fungi, and in several cases they have been postulated to be important for a successful colonization of the host plant. In the extreme case toxins define the host range of a fungus, i.e. all plant species, varieties or genotypes which are sensitive to a specific toxin are susceptible to the pathogen that produces it. These host-selective toxins (e.g. T-toxin in *Cochtiobolus heterostrophus*, HC-toxin in *C. carbonum*) have been studied in detail in the last years. Since this topic is covered by an excellent recent review (Walton 1996), it need not be treated here. For several other non-selective toxins more than indirect evidence now is available that they also contribute to pathogenicity of their producers, e.g. the *Fusarium* toxins trichothecenes and enniatin, and cercosporin from *Cercospora kikuchii* (see Table 1).

Hydrophobins, cell-wall-associated small hydrophobic proteins containing eight cysteines in a conserved pattern, have been identified in a variety of fungi. Their synthesis obviously is correlated with certain differentiation processes, e.g. formation of conidia and aerial hyphae, and they could very well be involved in the interaction of infection structures of pathogenic fungi with the host's hydrophobic surface (see review by Wessels 1996). A gene disruption approach has yielded different results

in two pathogenic fungi (see Table 1). The *mpg1* gene of *M. grisea* is obviously involved in the induction of appressoria formation; deletion leads to significantly reduced pathogenicity (Talbot et al. 1993, 1996). Talbot et al. (pers. comm.) managed to complement the *mpg1* deletion by hydrophobin genes from other fungi. On the other hand, disruption of a gene of *Ophiostoma novoulmi* coding for the hydrophobin ceratoulmin has no obvious effect on pathogenicity (Bowden et al. 1996). Hydrophobin-like proteins have been detected in other phytopathogenic fungi, e.g. in *Cladosporium fulvum* (Spanu 1996) and – unusual large ones (> 30 kDa) – in *C. purpurea* and *C. fusiformis* (Arntz and Tudzynski 1997; Moore, unpubl.); their importance for pathogenicity is under investigation.

Table 1 lists several other examples for analyses of putative pathogenicity factors by targeted gene disruption. Some of the results are interesting, since they might focus attention on new aspects of the complex host-pathogen interaction:

1. Disruption of the superoxide dismutase gene of *Glomerella graminicola* "drastically" reduces pathogenicity, without affecting growth and differentiation in axenic culture (Fang and Hanau 1996); this points to a role of this enzyme in detoxification of active oxygen species generated as plant defence reaction during the interaction. In *C. purpurea* recently the secretion of a catalase in planta has been demonstrated (and a corresponding gene has been cloned), which might have a comparable function (Garre et al., unpubl.). These enzymes protecting fungi against the oxidative stress in planta might represent pathogenicity factors.
2. Disruption of a gene for melanin biosynthesis in *Colletotrichum lagenarium* leads to albino appressoria and severe reduction in pathogenicity (Perpetua et al. 1996). This complements reports on non-pathogenic albino mutants, e.g. in *M. grisea* (Chumley and Valent 1990) and confirms the importance of mechanical pressure for the penetration of the plant cuticle.
3. Several putative pathogenicity/virulence/avirulence genes have been shown to be induced by nitrogen starvation, i.e. they must be under control of the nitrogen regulatory system.  
Astonishingly, disruption of the major nitrogen-regulatory gene of *M. grisea*, *nut1*, has no effect on pathogenicity (Froeliger and Carpenter 1996). On the other hand, studies using classic mutagenesis indicated that in *M. grisea* two other genes (*npr1* and 2) are involved in nitrogen regulation, and that they are essential for pathogenesis, e.g. they regulate expression of the *mpg1* gene (Lau and Hamer 1996). Nitrogen regulation of pathogenicity factors obviously is more complex than expected.

### 3. Methods: "Black Box" Approaches

Due to the complex nature of the mechanisms underlying host-pathogen interaction, isolation and functional analysis of predicted compatibility/virulence factors (see above) is not sufficient to gain a basic understanding of the systems. Therefore, the so-called "black box" approaches, looking either for a complete set of genes involved in the interaction using differential screening systems, or by random mutagenesis, have gained more importance and will dominate future research in this field. Therefore, the authors felt it adequate to present some of the major techniques (and results obtained with them) in detail. The **restriction enzyme-mediated integration (REMI)** procedure, first described in fungi for *Saccharomyces cerevisiae* (Schiestl and Petes 1991), is one of those new powerful methods, which offers the opportunity of randomly introducing tagged mutations into the fungal genome. Since transposon tagging has not been developed for routine production of marked mutants in fungi, REMI is the only method for insertional mutagenesis with high frequency.

REMI involves use of a transforming vector without major homology to the host's genome, carrying a selectable marker. However, the technique differs from a normal transformation in that the transforming DNA is linearized with a restriction enzyme, and a significant amount of the same restriction enzyme is added to the transformation mix. In the nucleus, the restriction enzyme cuts genomic DNA at its specific recognition sites which serves as sites of integration for the transforming vector. A survey of results obtained with REMI for different plant pathogenic fungi is given in Table 2.

The *tox1* locus of the maize pathogen *C. heterostrophus* which controls production of a polyketide phytotoxin was successfully tagged by REMI (Lu et al. 1994). Of 1310 transformants recovered, two produced no detectable T-toxin in culture or on plants. In each of these transformants, the *tox*<sup>-</sup> mutation tagged with the selectable marker (*hygB*), mapped at *tox1*, which was shown by crossing to a *tox1*<sup>+</sup> tester strain. Both mutants caused no symptoms on corn plants (Lu et al. 1994). In *M. grisea*, a fungus causing the blast disease of rice, the technique was successfully used for the identification of pathogenicity genes, host-specificity genes (avirulence genes) and genes involved in appressorium formation. Different types of integration patterns were observed among the transformants: single copy and multicopy integrations, tandem integrations at one or more sites and integrations with or without restoration of the specific restriction site. From about 600 REMI transformants examined, two sporulation mutants, one auxotrophic mutant and two mutants with reduced pathogenicity were recovered (Shi et al. 1995). Later on, the same group succeeded in isolation of more mutants blocked at critical steps in the sporulation pathway, by chemical and

Table 2. Examples of REMI mutagenesis in phytopathogenic fungi

Organism	Target	Number of transformants tested	Number of mutants	References
<i>Cochliobolus heterostrophus</i>	<i>tox 1</i> -Locus	1310	2	Lu et al. (1994)
<i>Ustilago maydis</i>	Pathogenicity genes	928	13	Bölker et al. (1995)
<i>Claviceps purpurea</i>	Pathogenicity genes	61	2	Voß et al. (1996)
	Auxotrophy genes	343	2	
<i>Botrytis cinerea</i>	Pathogenicity genes	93	5	Beermann and Tudzynski P. (unpubl.)
<i>Gibberella fujikuroi</i>	Gibberellin biosynthesis genes	1064	2	Linnenmannstons and Tudzynski B. (unpubl.)
<i>Magnaporthe grisea</i>	Pathogenicity genes	Approx. 1000	2	Shi et al. (1995), Shi and Leung (1995)
		5500	36	Sweigard et al. (1996)
<i>Alternaria alternata</i>	<i>tox</i> -Mutants	Several hundred	8	Akamatsu et al. (1996)



insertional mutagenesis (Shi and Leung 1995). From the five sporulation mutations derived from plasmid transformation (con1, con4, con5, con6 and con7), two (con1 and con7) are additionally blocked in the formation of appressoria. Pathogenicity on rice is totally lost in those mutants (Shi and Leung 1995).

In a recent update, Sweigard et al. (1996) reported that more than 5500 hygromycin-resistant *M. grisea* transformants were screened with a rapid virulence assay on barley. Thirty-six transformants with altered pathogenicity phenotypes were identified. Some of them induced no disease symptoms at all, while others produced reduced numbers of lesions; in some cases the lesions induced did not expand normally.

In the maize pathogen *Ustilago maydis* exclusively single-copy integration events with high frequency are generated by REMI. From 1000 insertion mutants analysed, 13 were unable to induce symptoms (plant tumours, anthocyan biosynthesis) when tested in planta (Bölker et al. 1995). To investigate whether the loss of pathogenicity is linked to the insertion event, the integrated plasmids plus flanking sequences were recovered from transformant DNA and used as disruption vectors for retransformation of the wild-type strain. In other cases, cosmid clones containing the flanking regions of the insertion were used for complementation of the pathogenicity defect.

Recently, first results on REMI mutagenesis for *Alternaria alternata* have been published (Akamatsu et al. 1996). This fungus is well known to produce a broad spectrum of host-specific phytotoxins. Two- to ten fold increases of transformation frequency were obtained in comparison with traditional transformation without restriction enzymes. Altogether, eight toxin deficient mutants were obtained.

Obviously REMI is a powerful method of obtaining mutants of a specific phenotype and of cloning the corresponding gene. Nevertheless it has its pitfalls. In general, experience with REMI in different fungi showed that on average 50% of the mutations have not been tagged. Segregation analysis, gene disruption using the rescued plasmids or complementation of REMI-mutants with a complete copy of the tagged gene (e.g. cosmid clones) should be used, therefore, to verify tagging before performing additional experiments.

In some fungal systems a standard transformation protocol (without addition of restriction enzyme) was used to generate insertional mutants. The plant pathogen *Cercospora cruenta* is well known to produce high amounts of the phytohormone abscisic acid (ABA). To characterize the role of ABA for plant-pathogen interaction, a mutant strain deficient in the synthesis of this hormone was isolated after standard transformation with a circular plasmid (Kitagawa et al. 1995). Using the same approach, a protein kinase gene was tagged in the bean pathogen *Colletotrichum lindemuthianum*, leading to a significant decrease in pathogenicity (Dufresne et al. 1996; see Sect. 4).

Besides these inactivation techniques, the study of **differential expression of fungal genes** during pathogenesis is becoming increasingly important to obtain a complete view of the plant-pathogen interaction. Since the last review (Tudzynski and Tudzynski 1996), several new genes involved in the infection process were identified by a variety of techniques.

In order to isolate *Botrytis cinerea* genes induced during its interaction with tomato, a comparative analysis of expression pattern in planta with that in axenic culture was performed by **differential display reverse transcription of mRNA (DDRT-PCR)** (Benito et al. 1996). For discrimination of in planta induced fungal genes from plant defense genes induced in response to the pathogen expression patterns of healthy tomato leaves and of tomato leaves infected with two different pathogens (*Phytophthora infestans* and *tobacco necrosis virus*) were included in the differential display analysis. Three *B. cinerea* cDNA fragments from genes showing enhanced expression in planta were isolated. Sequence analysis and comparison with the database did not reveal homology with any known genes (as is the case in several comparable approaches!) (Benito et al. 1996).

The same method has been used to isolate a set of clones derived from genes obviously expressed during gibberellin biosynthesis in *Gibberella fujikuroi* (Appleyard et al. 1995) and to identify putative pathogenicity genes involved in the *Phytophthora capsici*/pepper interaction. Several cDNA clones specific for different stages of infection were isolated and will be used for a functional test via disruption (Munoz Sanchez and Bailey Moreno 1996).

The method seems to be suitable even for molecular studies of mycorrhizal symbiosis between barley and *Glomus intraradices*. Using the differential display techniques, fungal genes that are differentially expressed in mycorrhizal roots were identified. Sequence analysis of two of the isolated fragments obtained from infected roots revealed homology to a DNA-binding transcriptional regulator and to a human protein interacting with the thyroid receptor (Delp et al. 1996).

Identification of fungal genes which are expressed exclusively or preferentially during growth in the plant may also be isolated by **differential screening of cDNA libraries**. To identify genes expressed during appressorium formation of *C. lagenarium*, differential cDNA screening was performed using mRNA from appressorium-forming conidia for library construction. Thirty-six cDNA clones which hybridized specifically to cDNA probes from appressorium-forming conidia were selected (Kuroda et al. 1996). Differential screening of a genomic library of the grey mold fungus *B. cinerea* was carried out by Prins et al. (1996) to identify fungal genes expressed during the colonization of tomato leaves. One of the genes identified codes for ubiquitin. This is especially interesting, since an ubiquitin gene is also induced during the interaction of

*Phytophthora infestans* and potato (Pieterse et al. 1991), and also in *M. grisea*; here the gene *ubi1* is expressed exactly 48 h after infection during asymptomatic colonization of rice tissue (not before or later); two other polyubiquitin genes have been identified which are expressed under stress (Talbot, pers. comm.) in *M. grisea*, the differential cDNA cloning approach is used by several research groups, and a whole set of genes has been identified (e.g. McCafferty and Talbot 1996); *mpg1* (see Sect. 2) has been one of the interesting genes obtained with this method.

Besides the fungal pathogenicity factors, the study of genes involved in fungal ectomycorrhiza development is of great interest. Martin et al. (1995) performed differential cDNA library screening and shotgun cDNA sequencing in order to identify symbiosis regulated fungal genes. Several differential expressed clones showed a significant amino acid sequence similarity to a family of secreted morphogenetic fungal proteins, the hydrophobins (Sect. 2). These studies together with cell fractionation and protein separation have emphasized the importance of fungal cell wall proteins during the early stages of the symbiotic interaction (Martin et al. 1995).

A similar technique, **subtractive hybridization** has been used to clone genes of *Phytophthora infestans* inducible in planta (Lehtinen and Saari-lahti 1996): The target cDNA from infected potato leaves was hybridized to excessive amounts of "driver" cDNA isolated from in vitro-grown mycelium. The remaining non-hybridized target sequences were amplified by PCR, cloned and sequenced. So far, no homology to other known genes has been found. Subtractive hybridization sscDNA screening was also used to isolate novel messages induced in the post-penetration phase in the *Phytophthora capsici* - *Capsicum annuum* pathosystem. Here, both susceptible and tolerant hosts recognize the pathogen quickly but differ in the response as measured by the abundance of specific gene transcripts. The power of this approach lies in identifying oligogenic resistance across genetically heterogeneous hosts by comparing post-penetration gene expression in tolerant versus susceptible plants (Vaughn et al. 1996).

Subtractive hybridization and differential cDNA screening were carried out with cDNA generated from germinating conidia of the obligate biotrophic fungus *Erisiphe graminis* (Justesen et al. 1996). Two cDNA clones which were highly expressed in germinating conidia on plant leaves code for small proteins of 249 and 251 aa residues and seem to have a function in development of *Erisiphe graminis* in planta.

To identify genes expressed during the early period of appressorium induction in *Colletotrichum gloeosporioides*, cDNA representing spores incubated in water were hybridized and subtracted from cDNA representing genes induced in spores treated with avocado wax (Kolattukudy et al. 1995). Only those clones which are uniquely expressed in appressorium-forming spores were cloned and sequenced. One of the appressorium-associated transcripts (*cap 20*) codes for a 22-kDa protein containing a signal sequence and two N-glycosylation sites, which are typical for secreted glycoproteins. Another transcript codes for a protein which was shown to be localized in appressorial walls. Gene replacement mutants incapable of expressing *cap 20* do not produce any symptoms on avocado and tomato fruits. RT-PCR analysis of infected fruit layers showed that *cap 20*

transcripts were found in the surface layer and in the infection front deep within the fruit (Kolattukudy et al. 1995).

A very promising approach (already successfully used for the identification of differentially expressed plant genes) has been used by Ruge and Schäfer (1994) for the identification of genes expressed by *Penicillium olsonii* on *Arabidopsis*: they designed a **promotor probe library** based on a vector carrying a promotorless GUS cassette (with *SphI* site including the start codon) and the acetamidase resistance gene as selectable marker and genomic *NlaIII* (CATG) fragments of 500–2500 bp length. Acetamidase-positive transformants were screened for GUS expression; from 750 transformants tested, 34 expressed the GUS gene, i.e. contained a genomic promotor fragment; two of these transformants expressed GUS only in planta. The corresponding genes have not yet been cloned and characterized, but this approach obviously is an attractive alternative method for the screening of differentially expressed genes.

Apart from the outlined differential screening techniques, which are designed to identify genes exclusively expressed in specific stages of infection, **large-scale cDNA sequencing** is now under way in several systems with cDNA clones generated from in planta-grown fungi. The completion of the nucleotide sequence of the 15.5 megabase genome of yeast changed the nature of fungal genetics from cloning single genes of interest to identifying how many genes act in concert to define the fungal phenotype under specific growth conditions. Large-scale cDNA sequencing has an immense potential to identify all expressed genes in fungal plant pathogens, including general "housekeeping" genes with homologues found in yeast. In this field, first results were reported with random cDNA screening for *M. grisea*, which has a genome size approximately three times that of yeast (Valent 1996).

Frequently this differential cDNA cloning approach led to the isolation of (putative) genes obviously specifically expressed during pathogenicity, but showing no homology to any published sequence, e.g. in rye tissue infected by *C. purpurea* (Oeser et al. 1996), in a culture of *Gibberella pulicaris* treated with the potato phytoalexin rishitin (Weltring 1996), or in the *B. cinerea* system (see above). Obviously, many so far unknown genes (having no homologues in the yeast genome) are involved in the plant-fungus interaction, and it will be especially interesting to learn more about their role in the infection process.

#### 4. Trend: The Role of Signal Chains in Fungal Pathogenicity

Signal transduction pathways enable eukaryotic organisms to respond to different environmental conditions, and they are of vital importance in cell-cell interaction systems like those involved in the interaction of

phytopathogenic fungi and their hosts. They have been studied in detail on the plant side, but also the fungal side now gains more and more attention (see Chen et al. 1996a). Fungal spores, in contact with their hosts, perceive the plant signals and consequently initiate gene expression that enables the pathogen to penetrate through the host plant barriers and to overcome the plant's defence reactions; therefore, the study of signal transduction pathways in fungi is the key for getting a complete view of the complex system of interaction between host and pathogen. Molecular cloning of members of signal chains and their functional study were therefore initiated for several fungus systems. One of the so far best analysed fungal signalling pathways will be described in more detail. Two genes encoding G-protein  $\alpha$ -subunits (heterotrimeric GTP-binding proteins serve as critical links in signal transduction pathways) from the chestnut blight fungus *C. parasitica*, *cpg-1* and *cpg-2*, were cloned (Choi et al. 1995). The predicted amino acid sequence for *cpg-1* was found to be 98% identical to a Gi-protein  $\alpha$  subunit from *Neurospora crassa* and 54% identical to rat and human Gi-protein  $\alpha$  subunits. Strains of the chestnut blight fungus are often infected with a so-called hypovirus resulting in reduced levels of virulence and modified gene profiles of the fungal host (Choi et al. 1995). This virus-infection-mediated hypovirulence is due to alterations of cellular signal transduction processes, thus exposing a role for G-protein-linked, cAMP-mediated signalling in fungal pathogenesis (Nuss 1996). The first indication of such an involvement came from Western analysis of protein extracts from isogenic virus-free and virus-infected *C. parasitica* strains revealing nearly undetectable levels of *cpg-1* accumulation in the infected strains. Gene disruption of *cpg-1* and *cpg-2* resulted in a set of phenotypic alterations in a *cpg-1*<sup>-</sup> mutant, similar to those associated with hypovirus infection, whereas disruption of *cpg-2* had no effect on virulence (Gao and Nuss 1996). Interestingly, both the *cpg-1* mutant and virus-infected strains failed to secrete detectable cellulase activity in comparison with virus-free virulent strains. Northern blot analysis revealed an increase in the accumulation of the transcript of a cellobiohydrolase gene (*cbh1*) after substrate (cellulose) induction. In contrast, induction of *cbh1* transcript accumulation was suppressed in an isogenic hypovirus-infected strain (Wang and Nuss 1995). Furthermore, similar to mammalian Gi  $\alpha$  subunits, *CPG-1* functions as a negative modulator of adenylcyclase and suggested a role for G-protein-regulated cAMP accumulation in hypovirus-mediated alteration of fungal gene expression.

A scheme of the working hypothesis of the role of the G-protein  $\alpha$ -subunit *CPG-1* in *C. parasitica* virulence is presented in Fig. 1. Newest data show that an important prerequisite for assembly of the heterotrimeric G-protein subunits and, therefore, for their functionality is the prenylation of the  $\gamma$ -polypeptide. Prenylation of  $\gamma$  is required for the  $\beta$   $\gamma$ -

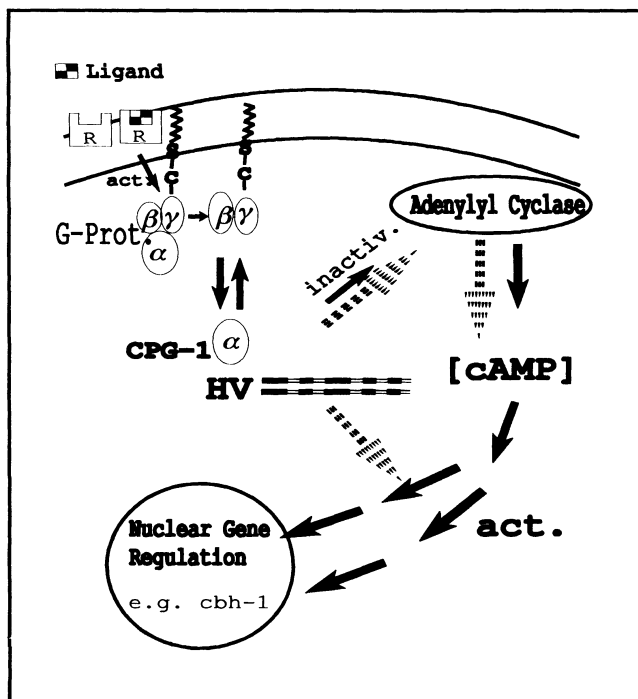


Fig. 1. Hypothetical scheme of the role of G-protein  $\alpha$ -subunit CPG-1 complex in *C. parasitica* virulence and the impact of hypovirus (HV) infection. R Receptor; act activated; G-Prot GTP-binding protein; *cbh-1* cellobiohydrolase 1. (Modified after Chen et al. 1996b)

subunit complex to interact with the  $\alpha$ -subunit. Additionally, interaction of a trimeric protein with a receptor, can be influenced by the specific isoprenoid (i.e. farnesyl or geranylgeranyl) attached to the  $\gamma$ -subunit (Zhang and Casey 1996).

Many plant pathogenic fungi produce specific penetration structures, the appressoria, when they have contact with the host surface. The question is which signals from the host plant specifically induce the formation of these structures in the fungus. For *Colletotrichum gloeosporoides* it could be shown that germination and appressorium formation of spores are induced by host surface wax of avocado fruits and by ethylene, the ripening hormone of fruits (Kolattukudy et al. 1995). Interestingly, among the surface wax of many plants tested only the host (avocado) wax induced germination and appressorium formation in this fungus. Both substances may induce the phosphorylation of 29- and 43-kDa proteins in the signal transduction. Involvement of protein phosphorylation in the germination and differentiation of appressoria was further

demonstrated with the protein phosphatase inhibitor calyculin A preventing the dephosphorylation of the 29- and 43-kDa proteins and resulting in the induction of appressorium formation.

Germlings of the plant pathogenic fungus *Uromyces appendiculatus* sense and respond to extracellular signals by undergoing a cell differentiation process resulting in appressoria formation. Recognition and mediation of extracellular signals is via transmembrane glycoproteins known as integrins (Correa et al. 1996), often exhibiting specific affinities to the tripeptide sequence Arg-Gly-Asp (RGD) found in several extracellular matrix components.

Besides G-proteins, protein kinases play an important role in signalling pathways. To initiate research on molecular communication in *Colletotrichum trifolii*, which is responsible for anthracnose disease of alfalfa, a kinase-encoding gene (*tb3*) was cloned (Buhr et al. 1996). The C-terminal catalytic domains of *tb3* and the serine/threonine protein-kinase, *COT1*, of *N. crassa* required for hyphal elongation and branching are highly conserved. Northern analysis indicated that *tb3* expression was highest 1 h after inducing conidial germination. The gene *tb3* complemented the *cot-1*<sup>-</sup> mutant of *N. crassa*, demonstrating the functional conservation of this kinase between a pathogenic and a saprophytic fungus (Buhr et al. 1996). From *Colletotrichum lindemuthianum*, which is responsible for common bean anthracnose, a serine/threonine kinase was isolated by screening for non-pathogenic mutants after integrative mutation via transformation of the plasmid pAN7-1 (Dufresne et al. 1996). The kinase gene was used to construct a disruption vector. Interestingly, nine out of 150 hygromycin-resistant transformants had a disrupted kinase gene and showed no symptoms after inoculation, demonstrating the close connection between the kinase gene and pathogenicity (Dufresne et al. 1996). Another example of involvement of protein kinases in signal transduction pathways that control infection process on host plants, was described for *M. grisea* (Xu and Hamer 1996). An *M. grisea* mitogen activated protein kinase (MAPK) was isolated and used for gene replacement experiments. Eleven *pmk1*<sup>-</sup> deletion mutants were isolated and none of them can form appressoria on inducing surfaces. Therefore, this protein kinase seems to be essential for development of infection structure and fungal growth in planta. Signal transduction elements involved in control of mating and pathogenicity have also been studied in detail in *Ustilago maydis* (Banuett 1995; Kahmann et al. 1996). One of the genes identified, *fuz7*, seems to have similar properties to the *pmk1* gene of *M. grisea*. It is potentially in the same signalling pathway, which might be a conserved way of sensing environmental changes and activating pathogenicity genes.

## 5. Perspectives

Our understanding of the mechanism and complexity of host-pathogen systems is rapidly increasing. As outlined in Section 3, the broad "black box" approaches – which have been very successful in revealing new aspects of the interaction systems – are expected to help us in developing better, more realistic models, therewith giving us the clues to develop new, specific plant protection strategies.

An especially interesting future aspect of the progress made in the development of molecular techniques for analyses of fungi is their possible application to systems, which so far had been recalcitrant to genetic and physiological analysis, the mycorrhizal and endophytic fungi (see recent reviews by Gianinazzi-Pearson 1996; Schardl 1996). Preliminary data obtained with such systems indicate that the mechanisms involved are not essentially different from those observed in pathogenic interactions. The comparison will help us to understand the mechanisms deciding whether a plant-fungus interaction will be a pathogenic or a mutualistic one.

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## **Population Genetics: Genetic Analysis and Modelling of Natural Populations**

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### **1. Introduction**

Gaining some insight into evolutionary processes by studying changes in the genetic composition of populations or species is one of the main purposes of population genetics within evolutionary biology. If questions about plant or animal breeding arise the genetic structures will be analysed with respect to the economic capability of those lines, populations or varieties concerned. Nowadays, population genetics has received special attention because of its various interrelationships with other scientific areas, in particular ecology. For example, estimating gene or genotype frequencies in time and space will give some results on the adaptiveness of a population to ecological constraints and its capability of surviving in a specific environment. Such analyses establish links between genetics and ecology, both significant subjects of evolutionary research.

The utilization of biochemical and especially molecular markers has opened up a promising field for an additional analysis of the mode of operation of evolutionary forces. The impact of these factors may be analysed directly in natural populations and ecological problems can be handled experimentally, including genetic aspects. This field developed parallel to the technical progress in gene technology and is often called molecular ecology.

Besides the analysis of the genetic architecture of populations and their stability, questions concerning dynamic processes and predictability of changes within and between populations are of significant relevance. Mathematical methods like modelling or especially computer simulation become more and more accepted. The actual development of populations as represented by the variation in gene and genotype frequencies or just by population sizes is often difficult to assess as plenty of biotic and abiotic forces will influence it. Quantification of these factors is of great importance in modelling population dynamics in applied ecology, e.g. in conservation genetics or in plant protection, especially for forecasting the development of natural populations.

Making use of some examples from molecular ecology and disease epidemics this chapter gives an overview on recent approaches to the genetic analysis of natural populations and the modelling of their dynamic behaviour in time and space with special emphasis on a new class of models, the cellular automata.

## 2. Molecular Ecology

### a) Tools for Genetic Analysis

Most natural populations are regional. This means that as a result of distance or geographical barriers, the isolation of individuals leads to specific genetic, and as a consequence sometimes to different morphological, patterns. In addition, evolution of a population is strongly influenced by the effective size of the population. Small populations are often threatened by inbreeding depression which decreases the genetic variation and heterozygosity. Genetic diversity is a necessary prerequisite for any future adaptive change or evolution, i.e. species that lack adequate genetic variation are at greater risk of extinction; therefore conservation of genetic variation is of major concern in terms of further uses in plant breeding, for example the introgression of desirable genes from wild species into cultivated species.

Three major types of characters have been used to estimate levels of variation: morphological, biochemical and molecular markers. Analysis of morphological variation is suitable when a rapid estimation of variability is needed or where biochemical surveys are impractical. In some cases phenotypic characters are ecologically adaptive and are assumed to indicate genotypic variation, local differentiation or ecotypes. However, phenotypic plasticity is common in plants and has to be considered.

Until recent years, isozyme electrophoresis was the technique most widely used for examining genetic variation in plant populations. Isozymes were also used to identify species, cultivars and hybrids, and to delineated clones as in broad bean (Bassiri and Rouhani 1977), barley and oats (Almgard and Norman 1970), corn (Goodman and Stuber 1980) and soybean (Blogg and Imrie 1982). In some cases, however, species-diagnostic alleles cannot be found, because relatively few loci and alleles have been used. This is due to technical difficulties, because only soluble enzymes can be analysed, and in terms of ease of enzyme extraction and a limited number of enzyme systems in most species there are insufficient numbers of allozyme markers available for examining large proportions of the genome. In addition, only nucleotide differences in genes that lead to changes in amino acid composition can be detected; therefore these genes may not be representative of the genome in general (Schaal et al. 1991). Nevertheless, allozyme analysis is relatively fast, inexpensive and straightforward and methodologies have been developed for many kinds of plants (Hamrick 1989). In most cases allozyme variation does seem to reflect the overall level of genetic variation within populations. Also, most often there is a correspondence between morphological and allozyme data. Hamrick and Godt (1989) discov-



ered that in an allozyme analysis of 450 plant species an average of 50% of allozymes are polymorphic within a plant population. Furthermore, widespread species exhibit greater genetic diversity than local species do. In addition, genetic diversity is greater at both the population and species level for outbreeders than for selfers.

When analysing plant populations, especially if only a limited number of polymorphic isozyme markers is available, other molecular techniques such as restriction fragment length polymorphism (RFLP) or random amplified polymorphic DNA (RAPD) markers should be included.

RFLPs provide an efficient method for estimating genetic variation among genotypes (Botstein et al. 1980; Burr et al. 1983). RFLP markers are detected by hybridizing labelled DNA clones to Southern blots of DNAs digested with a certain restriction enzyme. The variation is detected by determining the presence or absence of 4–6 bp long restriction sites specific for a particular restriction endonuclease.

Two techniques related to RFLP markers surveys are variable number of tandem repeats (VNTR) analysis (Nakamura 1987) or investigation of highly variable region (HVR) markers (Jeffreys 1987).

Variable numbers of serial repeats of a core DNA sequence of 15–35 bp length are dispersed throughout the genome in all eukaryotes. These core sequences are called minisatellites or microsatellites (1–4 bp long). The number of repeats in a particular minisatellite can vary among individuals of a given population. DNA samples are digested with a restriction enzyme that recognizes restriction sites flanking a specific VNTR locus. A probe from human minisatellite DNA or tandem repeats from bacteriophage M13 DNA, representing the core sequence of the VNTR, is used for detection of length variation. The resulting banding pattern is highly polymorphic. In many cases this banding patterns is characteristic of a certain individual and is therefore called "DNA fingerprint" (Jeffreys et al. 1985). VNTR markers are nowadays widely used for the detection of fingerprints, because the method is relatively simple and reliable, and also because synthetic oligonucleotides such as (GATA)<sub>n</sub> or (GACA)<sub>n</sub>, which reveal highly polymorphic patterns (Weising et al. 1989, 1990, 1991), are available. Some of the most useful applications of minisatellite analysis in plant biology are the characterization of cultivars (Nybom et al. 1989; Nybom and Hall 1991) and inbreeding lines (Mösges and Friedt 1994) or paternity analysis (Nybom and Schaal 1990). This technique, however, is methodologically complicated.

A more simple method of population studies is described by the use of RAPD markers. This novel type of genetic marker was first described by Welsh and McClelland (1990) and Williams et al. (1990).

RAPD markers are generated by the amplification of DNA fragments with single primers of arbitrary nucleotide sequence. This reaction is non-radioactive, easy to perform and requires only small quantities of genomic DNA. The genetic tests are based on the polymerase chain reaction (PCR). To perform an RAPD assay, a random primer of arbitrary sequence is mixed with genomic DNA in the presence of a thermostable DNA polymerase and a suitable buffer, and then is subjected to temperature cycling conditions typical for the PCR. The products of the reaction depend on the sequence of the primer, as well as the reaction conditions. At an appropriate annealing temperature during the thermal cycle, the single primer binds to sites on opposite strands of the genomic DNA that are

within an amplifiable distance of each other (within a few thousand nucleotides), and a discrete DNA segment is produced. The presence or absence of this specific product, although amplified with an arbitrary primer, is diagnostic for the oligonucleotide-binding sites on the genomic DNA. In practice, the DNA amplification reaction is repeated on a set of DNA samples with several different primers, under conditions that result in several amplified bands from each primer. It has been reported that between 40 and 80% of primers can give polymorphic markers depending on the techniques used (Hedrick 1992).

RAPD analysis has some disadvantages. The genomic origin of fragments and the sequence homology of bands with similar mobility in a gel are not known. Different RAPD bands having the same molecular weights can comigrate in the gel and therefore not be detected (Riedy et al. 1992; Lamboy et al. 1994; Smith et al. 1994). Because only a small amount of DNA is necessary for amplification, the RAPD analysis is prone to artefacts caused by contamination from foreign DNA, e.g. fungal DNA on plant tissues, cross-contamination during DNA extraction or contaminated reaction tubes. Also, RAPD markers behave as dominant markers and the only character that can be scored is absence or presence of a DNA fragment.

RAPD markers are increasingly used for taxonomic identification, paternity and kinship testing, linkage analysis and population genetics (for reviews see Hadrys et al. 1992; Bowditch et al. 1993; McClelland and Welsh 1994; Sobral and Honeycutt 1994; Welsh and McClelland 1994).

The amplified fragment length polymorphism (AFLP) technique is based on the selective PCR amplification of restriction fragments from a total digest of genomic DNA.

Two steps are involved: (1) digestion of DNA with restriction enzymes, followed by ligation of oligonucleotide adapters to the DNA fragments, (2) selective PCR amplification of desired restriction fragments by using the adapter and restriction site sequence as target sites for primer annealing. The selective amplification is achieved by the use of radioactive-labelled primers that extend into the restriction fragments, amplifying only those fragments in which the primer extensions match the nucleotides flanking the restriction sites. This method can obtain up to 100 different fragments or monomorphic and polymorphic DNA markers, which can be detected on denaturing polyacrylamide gels.

AFLP analysis produces ideal markers for a wide array of genetic investigations, because the method is comparatively time- and cost-saving, providing highly reproducible and informative genetic data. AFLP markers can be treated in the same way as RFLP markers and are useful to analyse genetic diversity in breeding lines (Vos et al. 1995), e.g. of rice (Mackill et al. 1996), wild bean (Tohme et al. 1996) and fungi (Majer et al. 1996). They were also used for extending RFLP linkage maps in sugar beet (Schondelmaier et al. 1996) and barley (Becker et al. 1995) and segregation analysis in soybean (Maughan et al. 1996).

## b) Assessment of Environmental Impact: An Example

Geographical variation is often the result of a physical adaptation to variable ecological conditions. Plant populations with a widespread geographical distribution often show a range of phenotypic variations, which can be due to phenotypic plasticity and/or genetic variation. Locally adapted genotypes, described as ecotypes, are correlated with specific habitats; however, each population is a unique realization of the genotype-environment interaction. Differences between populations may be response to unusual edaphic and climatic conditions, e.g. soil types, rainfall or fertiliser treatments. Furthermore, changes occurring gradually rather than abruptly over space will form genetic divisions, which are known as clines.

Genetic variation is the most important determinant for geographical and ecological adaptation of populations. However, phenotypic plasticity can often conceal the true extent of genetic variation, especially when analysing populations growing along resource gradients which generally cannot fully compensate for low genetic variability in a heterogenous environment. In terms of reducing plasticity, however, genetic variation will contribute to the fitness of a population.

The Sahel zone represents the transition zone between the arid Sahara and the moist tropical savannas of Africa (Fig. 1). It is known for decreasing rainfall, an increasing population, poverty and scarce natural resources. The high contribution of African species to the tropical pasture and fodder genepool is emphasized by Le Hourèou (1991). Although considerable data on genetic diversity in the gene pools of the major crops have been collected already – crop species like millet, wheat and sorghum (Devos and Gale 1992; Tao et al. 1993; M'Ribuh and Hilu 1994) – relatively little attention has so far been given to numerous wild fodder species (McCusker and Toll 1991; Huff et al. 1993). Their value and potential are poorly known and they are at risk of genetic erosion. Thus, a need to strengthen the scientific base of forage plant genetic diversity conservation was recently recognized (FAO 1996).

A first attempt to analyse forage plants from the Sahel zone has recently been carried out, using biochemical and molecular genetic methods (Kusserow et al. 1997). Some 300 and 80 accessions, respectively, of two main forage plants, *Brachiaria* sp. and *Zornia glochidiata*, were collected from 17 different sites throughout the Sahel zone. The region under study extended from the Lake Chad region in the east to Mali in the west and the maximum distance between collection sites was about 2000 km (Fig. 2). Samples were taken over three successive years (1994–1996) during rainy seasons (July–September), mainly from roadsides with a sampling area of 10–20 m long and 2–5 m wide.

For the purpose of genetic conservation the following questions should be asked: (1) Are there differences in the genetic patterns of

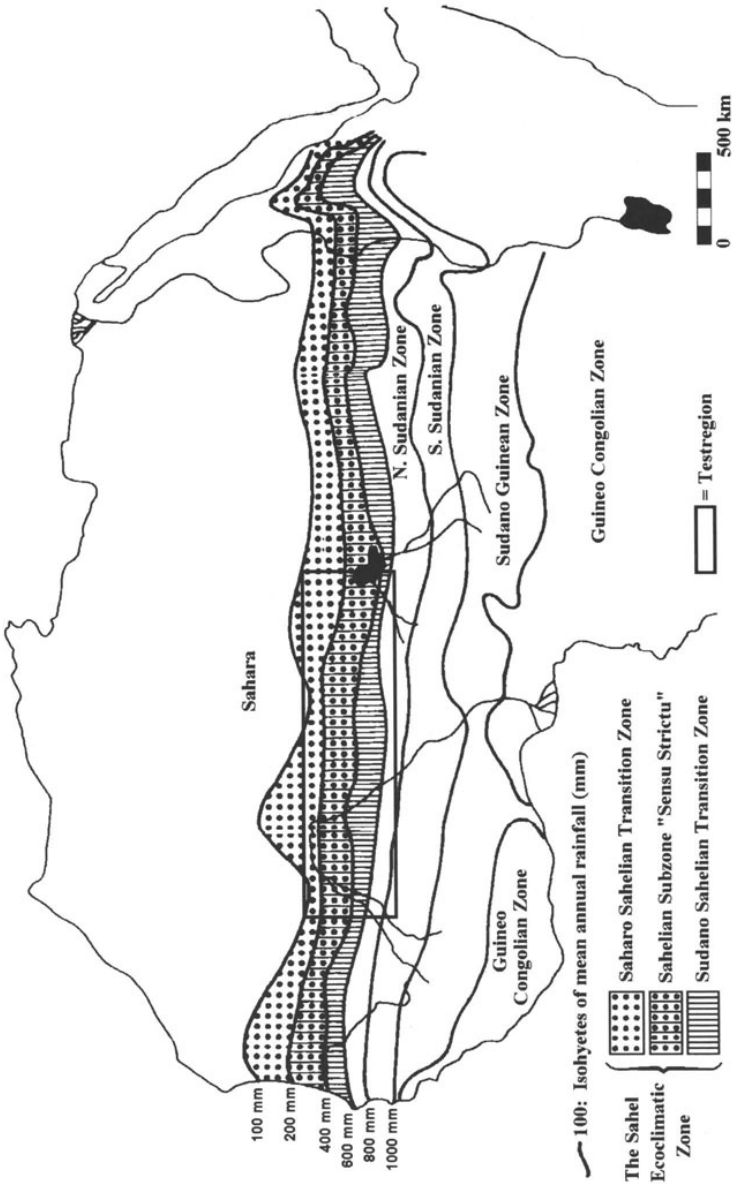


Fig. 1. Map of West Africa, including the region referred to as the Sahel zone

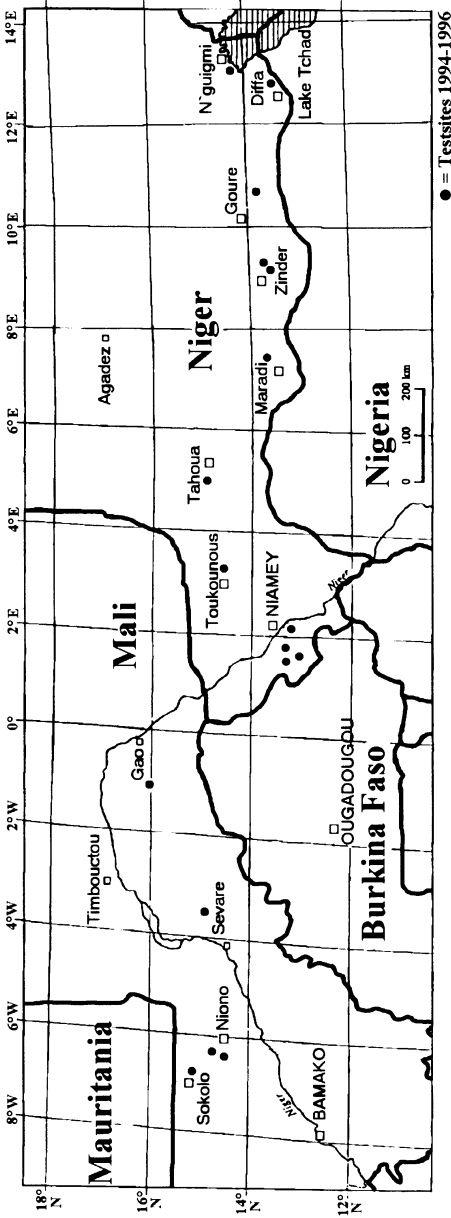


Fig. 2. Map of ecoclimatic zones in the Sahel zone. (Changes from Le Hourèou 1989)

samples collected from different ecoclimatic and geographical test sites? (2) Are there associations between genetic patterns and ecoclimatic zones? (3) Are there cyclic variations in the patterns of genetic diversity over longer periods of time?

This study should help to establish guidelines for the conservation of certain kinds of germplasm in dry areas by identifying suitable sources of genetic variability. Further work might show how the genetic structure of plant populations may be affected directly by favourable/unfavourable climate periods or via their effects on soil characteristics, for example.

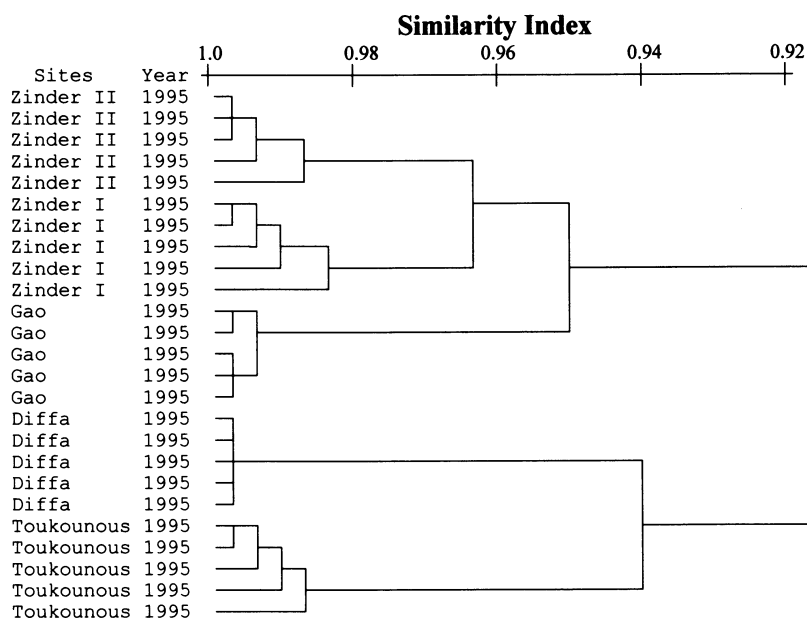
Using RAPD markers, all 300 samples from *Brachiaria* could be determined to their corresponding species. Five different species were common in the study area: *B. ramosa*, *B. lata*, *B. xantholeuca*, *B. nidulans* and *B. orthostachys*. Some species are limited to different ecoclimatic zones (Fig. 2; described by Le Hourèou 1989). Individuals from *B. nidulans* and *B. orthostachys*, for example, are only present in one ecoclimatic zone, i.e. the Sahelian zone which is characterised by 200–400 mm rainfall per year, while individuals from *B. xantholeuca* were dispersed in the Sudano-Sahelian transition zone with 400–600 mm rainfall per year.

Genetic variation at the species level shows different patterns. While *B. orthostachys* showed only minor intra-species variation, in *B. xantholeuca* high genetic diversity was found. For *B. xantholeuca*, only one test site in Mali could be clustered separately, probably due to geographical isolation. In addition, a comparison of individuals from one test site collected in 2 successive years with different rainfall patterns showed specific band patterns as a possibly environmental effect. However, results must be interpreted very carefully, because two limitations have to be considered.

First, it is generally difficult to estimate the climatic influence, because only a few weather stations near the test site exist and a deviation of more than 100 mm rainfall per year is possible even at two closely located sites, e.g. at Niamey Airport and City. Second, only 2 years were compared, but more years have to be taken into account to verify results. However, molecular genetic methods are sensitive enough to detect minor changes in the composition of genetic structure of populations.

In *B. nidulans*, all individuals were identified according to their corresponding test sites, although only five samples from each site were analysed (Fig. 3). All five ecotypes belong to the same ecoclimatic zone but are geographically separated. Results from the second forage plant *Zornia glochidiata* showed the same genetic effects when comparing 2 years. At two test sites 2 years could be discriminated.

This analysis gives a first insight into the genetic structure of two forage plants, indicating correlations between environmental factors and the genetic structure of populations. Only populations able to rapidly



**Fig. 3.** UPGMA dendrogram of five test sites of *B. nidulans*. Each site is clearly separated from the others. Two test sites in Zinder show small differences according to their close proximity (5-m distance, other side of the track)

adapt to ecological changes will survive and propagate in extreme climatic environments, having high genetic diversity or phenotypic plasticity. Especially in the Sahel zone, populations are sometimes very small and are extremely threatened by extinction due to overgrazing and changing agronomic management. One of the major issues in conservation biology is thus the maintenance of all existing levels of genetic diversity, because diversity is a necessary prerequisite for any future adaptive change or evolution.

### c) Consequences of Gene Conservation and Plant Breeding

The genetic resources of agronomic plant species and their wild relatives are rapidly decreasing. Global food production is dominated by a small number of plant species and relatively few cultivars, e.g. rice, wheat and maize are the three main crops. However, plant breeders have always been dependent on genetic material from traditional land races, which contain genes profitable to locations with different environmental conditions. The extinction of land race genes is increasing due to modern agricultural practices, so the conservation of biological diversity is a key

prerequisite for the future. The molecular identification of useful relevant genes from the conserved gene pools will become a particularly important tool in the future. There are many reasons for the rapid erosion of plant genetic resources available to humans, in particular the introduction of new and uniform cultivars, deforestation, urbanization and changes in agricultural management. In order to maintain remaining plant genetic resources, biotechnology can be very convenient for efficient collection, especially in providing information on the available genetic diversity of a particular region. In recent years, therefore, biochemical and molecular genetic markers have become very useful in assessing genetic diversity of populations as well as contributing to successful collection and analysis of problem species, such as vegetatively propagated species (Hodgkin and Debouck 1992, Withers 1994). Sampling methods must be based on sound scientific principles to recover maximum genetic diversity through sampling of different forms or geographical sites (Falk and Holsinger 1991; Guarino et al. 1995). There are two approaches to the conservation of plant genetic resources: *ex situ* and *in situ*.

*Ex situ* conservation is carried out in various ways. Under optimal conditions, seeds can be stored for several years without the need to regenerate them (Ellis 1985). Nevertheless, seed viability depletes over the years and regeneration is necessary to maintain genetic variation in a particular seed population. However, some changes in genetic structure after regeneration are inevitable due to genetic drift, genetic shift, selection and outcrossing (Breese 1989; Ramanatha Rao 1991). Recalcitrant seeds are difficult for *ex situ* conservation, because they require different storage management, i.e. cryopreservation for long-term storage (Chin and Pritchard 1988) or ultra-drying and storage at room temperature. Plants where seed conservation is not recommended for a long-term storage are generally conserved in field genebanks, e.g. cassava, potato, apple and banana. For some species, *in vitro* conservations is the only possibility, especially of germplasm of vegetability propagated material or recalcitrant seeds. A main problem in tissue culture is genetic instability due to somaclonal variation and, in addition, the duration of storage as tissue is limited. Some success can be gained by reducing growth of cultures through reduction of storage temperatures, changes in culture medium or mineral oil overlay (Withers 1991); however data on long-term effects of slow growth is limited by now. Changes in genetic composition in tissue cultures, e.g. in banana, can now easily be analysed by molecular markers, e.g. RAPDs (IBPGR 1993). Cryopreservation of *in vitro* cultures provides some advantages over other methods, especially in terms of long-term physical and genetic stability (Engels 1993) as well as quick and easy access. Another method for conservation of plant genetic resources is the use of DNA libraries, including genes useful for breeding programs. Good results have been achieved using biotechnological methods, especially in improving resistance against pests and diseases and tolerance to abiotic stresses by introgression of the respective genes into cultured plants.

Another approach to maintaining plant genetic resources is *in situ* conservation. *In situ* conservation is defined as the maintenance of ecosystems and natural habitats, and can be carried out either in the natural habitat of the plant or under controlled conditions, for example in nurseries or on farms, depending on the material under consideration (Falk



and Holsinger 1991; Ndambuki 1991; Guarino et al. 1995). This type of conservation is dynamic compared with *ex situ* conservation and provides the species or populations with an opportunity to evolve under natural conditions. Specific areas are designed as gene conservation areas, where wild relatives of crop species can be protected and sampled by scientists. On-farm management uses the help of farmers in growing of especially recent crop species as well as old cultivars and land races.

### 3. Disease Epidemics

Pathogens are naturally present in most crops as endemic diseases at low densities. In natural ecosystems the host plants and the pathogens are interacting populations, and each possesses high genetic variability (Marshall 1977; Day 1978). On the time scale, this interaction is described as co-evolution between host plants and pathogens, where the frequencies of host and pathogen genotypes are the result of the balance between host, pathogen and environmental factors (Burdon 1993; Hovmøller et al. 1997). This means that the genetic composition of a pathogen population is under selection pressure from the host population and vice versa. Additionally, both populations are subject to selection pressure from environmental factors. An alteration in environmental conditions, such as climatic changes, may favour the disease and eventually produce epidemics, resulting in severe damage to the host plants and putting at the same time strong selection pressure on the host. Only sufficiently resistant host genotypes will survive and in turn increase the selection pressure on the pathogen.

Dramatic changes may arise from human intervention such as the transformation of wild to cultivated systems. These agricultural systems are characterised by techniques such as plant breeding, fertiliser applications and various disease control measures and by the presence of large areas of crops with identical or closely related host genotypes. All of this will strongly affect the pathogen population dynamics (Hovmøller et al. 1997), while selection force on the host population through the pathogen may be neglected, because it is controlled by man.

#### a) Plant-Pathogen Interactions

The interaction between host and pathogen was first described by Flor (1956). He proposed the gene-for-gene hypothesis which states a specific reaction between two genes, namely, on the pathogenic side an avirulence gene may react with a resistance gene on the host side. If the host is resistant, the pathogen is by definition avirulent; on the other hand if the pathogen is virulent, the host is susceptible (Day 1978). The concept

of gene-for-gene interactions to describe pathogenicity has been applied to a wide variety of pathogens, namely to fungal, viral and bacterial pathogens as well as nematodes and insects (Christ et al. 1987). Based on Flor's hypothesis, Vanderplank (1963) developed a broader model describing the interaction of plant and pathogen. He distinguished between vertical resistance, where only a single gene is involved, and horizontal resistance which is polygenic. If the pathogen is virulent, which occurs in the absence of a single avirulence gene, the polygenically controlled aggressivity determines the degree of the infection.

For example, the plant-pathogen interaction within the pathosystem powdery mildew (*Erysiphe graminis*) and barley (*Hordeum vulgare*) is determined by a single major (vertical) resistance gene in the host, which causes a hypersensitive death of the host cell but can be overcome by specific virulence genes in the pathogen.

The incidence of compatible and incompatible interactions with different hosts containing different resistance genes enables the classification of the pathogen population into distinct physiological races (Honecker 1934; Nover 1957; Welz and Kranz 1987). The introduction of a variety containing a new vertical resistance gene will be a strong selection factor to the pathogen population. In this case the mutation rate (Hermansen 1980; Hollomon et al. 1984; Torp and Jensen 1985; Sherwood et al. 1991), the extent of sexual reproduction (Welz and Kranz 1987; Brown and Wolfe 1990) and the strength of selection will determine the time period, until the pathogen population has regained a sufficient number of individuals virulent towards the new variety to make the implemented disease control ineffective.

Horizontal resistance, also known as partial resistance, results from the interaction of many genes and generally reduces the lesion size, the infection efficiency or the spore production of the pathogen. Horizontal resistance is considered more durable than vertical resistance. The interaction between the pathogen and a horizontally resistant host is non-specific in contrast to the specific gene-for-gene interaction in vertical resistance. Non-specific interactions means that the effectiveness of resistance is the same on genetically diverse pathogen individuals. There may, however, be variation in aggressivity within the pathogen population, which enables some individuals to grow better than others, i.e. to possess a shorter latency period, a greater infection efficiency, a larger lesion size or higher spore production.

In most host plants the specific vertical resistance and the non-specific horizontal resistance occur together, and both types of resistance may be more or less combined in such a manner that the vertical resistance gene is modified by polygenes in the background. Another view is that polygenically determined resistance results from resistance genes that have been matched by virulence genes. In order to decide between the two mechanisms one has to know whether the host-

pathogen interaction is specific or non-specific (Leonard 1987). Regardless of the nature of polygenic resistance, the selection force originating from the host derives from a combination of both resistances. This situation necessitates the pathogen not only to select a compatible virulence genotype (race), but also to act highly aggressively. Other environmental factors such as temperature might be neglected at this point. The question concerning the ability to survival may introduce the term fitness as a synonym for the selective or reproductive value of genotypes. Fitness either may be estimated as an absolute value (the number of progeny per parent) or is determined relative to some reference genotype (Crow and Kimura 1970). For the powdery mildew, as for most biotrophic pathogens, absolute fitness is expressed by an absolute value, e.g. the spore production, lesion size, infection efficiency or the duration time of the latent period, all of which are closely related to aggressivity. The fitness of genotypes can also be estimated relative to a standard by recording their frequency changes of genotypes during a period of interest. This is usually done by observing virulence frequencies within the powdery mildew population at different times and calculating the virulence dynamics from these data (Welz 1987).

If the genetic resistance of the host does not provide sufficient protection, supplementing fungicide must be applied alongside. However, the pathogen population may overcome the effect of fungicides by the development of resistance against it.

The genetic basis of fungicide resistance is not yet known in detail. In general, a distinction is made between single-gene and polygenic control of fungicide resistance. Single-gene control of the trait results in a discrete, discontinuous distribution of the fungicide sensitivity within the pathogen population, while a continuous distribution of the sensitivity is caused by a set of interacting genes, which do not have a significant effect individually. In the first case, disease control is lost, and the fitness costs of resistant individuals are expected to be neglected. The fungicides used here are described as high-risk fungicides. In the latter case, control is above all still possible and it is expected that fitness costs of resistant individuals are high. The fungicides used here have a low or moderate risk. Ethirimol and triazol fungicides used prevalently to combat powdery mildew fungus are usually classified as polygenic with low or moderate risk. However, more recently a discrete, discontinuous distribution of fungicide sensitivity for both agents was reported (Brown 1996).

Independent of the underlying genetic mechanisms of the fungicide resistance, the sensitivity of the pathogen to a specific fungicide, may be measured by the median effective dose ( $ED_{50}$ ); this parameter shows high genetic variation within most pathogen populations (Fig. 4). Beside manmade host resistance, fungicides are a major selection factor.

The parameters described for the study of plant pathogen populations derive from host-pathogen interactions and the fungicide response. For each, the pathogen population has high genetic variability, allowing the study of the genetic composition of pathogen populations. Additionally,

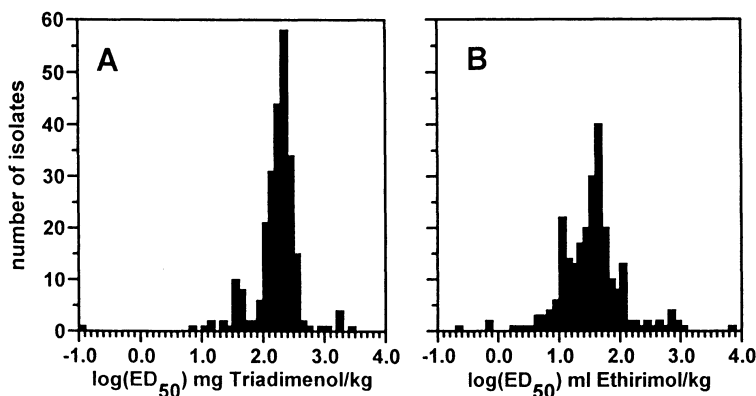


Fig. 4 A,B. Distribution of the logarithm of effective doses ( $\log ED_{50}$ ) of triadimenol (A) and ethirimol (B) in a population of *Erysiphe graminis* f. sp. *hordei* collected from untreated barley plants. (Pons and Hau 1992)

genetic markers (see Sect. 2.a) are available for the analysis of the pathogen population, specifically for the analysis of the powdery mildew example. Significant research was done, for example by Brown et al. (1990), Giese et al. (1990), Koch and Köhler (1990), Koch (1993) and McDermott et al. (1994). The major difference between the character's virulence and fungicide sensitivity and the DNA-based markers is that virulence and fungicide sensitivity might be subject to strong selection by host resistance and fungicides, while DNA-based markers, on the other hand, appear at least in part selectively neutral (McDonald and McDermott 1993; Wolfe and McDermott 1994). Selective neutral markers are the basis for an unbiased estimation of the potential for the genetic change in pathogen populations and an accurate measure of their genetic structure. For a detailed discussion see McDonald and McDermott (1993).

#### b) Dynamics of Gene Frequencies During Epidemics

One assumption in the study of gene frequency changes in pathogen populations is that genetic variation in a character, and character differences, are stable over time. To determine the formal genetics of an observed character, classic genetic analysis requires that strains differing in the character are able to be crossed. To some extent this may be difficult for obligate biotroph parasites such as powdery mildew (Barrett 1987). Nevertheless, some crosses of powdery mildew isolates have been performed using isolates differing in characters such as fungicide sensitivity (Hollomon 1981; Brown et al. 1992) or DNA markers (McDermott et al.

1994). The results show segregation among these characters in the progeny population, which might be a clear indication of their inheritance.

Investigation of genetic variation of powdery mildew fungus characteristics has been performed extensively by virulence surveys within different European countries and across Europe (for reviews see Jørgensen 1991; Zeller and Fischbeck 1992). With some exceptions, e.g. Welz et al. (1990), Huang et al. (1994) and Pons et al. (1996), relatively less attention was devoted to the analysis of dynamics of genotypes within single fields during disease epidemics. Moreover, Hovmøller et al. (1995) observed virulence dynamics within a limited agricultural area with changing frequency of host resistance genes. The variation of fungicide sensitivity was also studied in European surveys, but additionally in field experiments, mostly to investigate the effectiveness of application strategies in preventing buildup of fungicide resistance (e.g. Wolfe et al. 1986; Brent et al. 1989; Hau and Pons 1996). Investigations of the variation of DNA markers in large population samples of powdery mildew have been done by Brown et al. (1990), Koch (1993), Brändle (1994) and Wolfe and McDermott (1994). Koch (1993) analysed isolates of a European survey for RFLP-marker variation; however, examination of molecular genetic variation in populations of powdery mildew during an epidemic process has not yet been carried out.

The following factors might contribute most to changes in gene frequency in the populations of interest: population size, mutation, migration, selection and sexual reproduction. McDonald and McDermott (1993) gave a detailed overview of the relative importance of the various factors for plant pathogenic fungi.

For powdery mildew, finite population size can be ignored because agriculturally important pathogens exist in extremely large numbers. The importance of sexual reproduction is controversial, but can be disregarded because of the asexual reproduction in the haploid phase during a single epidemic. Mutation is the source of variation and difficult to estimate, but will only be significant over a long time scale and might not be important if one observes gene dynamics within single epidemics. Migration is one of the most neglected areas of gene frequency dynamics, but can be considerable in the biology of many pathogens (Barrett 1987). Throughout a powdery mildew epidemic, however, migration will only be important when a field is initially infected during the esodemic process (Kranz 1996). During the exodemic phase, the epidemic continues and all of the new infections are derived from the initial infections. In this phase the influence of migration can be ignored (Kranz 1996).

While all of the above-mentioned factors may have relatively little influence on the gene dynamics within a powdery mildew population during a single epidemic, selection might be the most powerful force (Østergård and Hovmøller 1991). All pathogen genotypes which are unable to propagate on resistant or fungicide-treated host plants will not survive (Hovmøller et al. 1997). Therefore, estimating selection coefficients for gene frequency dynamics during a selection process is the same as esti-

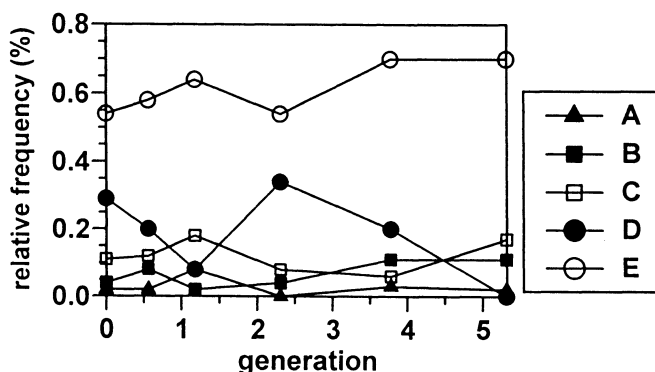


Fig. 5. Changes in relative frequencies of genotypes of *Erysiphe graminis* f. sp. *hordei*, differing in fungicide response to triadimenol and ethirimol during successive samples in an untreated barley field. Relative fitness for genotype E was standardised to 1. Estimated relative fitnesses of remaining genotypes are 0.80 for D, 0.95 for C, 1.1 for B and 0.98 for A

inating the fitness of the gene of interest. Leonard (1969) first introduced a simple model to describe the gene frequency dynamics in successive samples by estimating relative fitness. Bronson and Ellingboe (1986), Østergård (1987), Welz (1988), Pons and Hau (1991) and Pons et al. (1996) have extended, applied and discussed that model.

Figure 5 displays changes in relative frequencies of five genotypes of powdery mildew, differing in fungicide response to triadimenol and ethirimol, from successive samples in a barley field plot, connected with the estimated relative fitness. The relative fitness from one of the genotypes was set to 1, so that the estimated values of the other genotypes have to be interpreted in relation to the standardised one.

Along with this simple model, other models were developed to study virulence dynamics in multilines and variety mixtures with different resistance genes in mixture components (Barrett 1980; Østergård 1983), and, in addition, for analysing survey data with multi-locus interactions among virulence alleles, considering selection through combination of host resistance genes (Østergård and Hovmøller 1991). Emphasising gametic disequilibrium among virulence alleles, these models were discussed in detail by Hovmøller et al. (1997).

### c) Disease Modelling

Different models for plant disease epidemics are available, the most common ones being growth curve models, which were introduced from other scientific areas, such as ecology (Madden 1980). Practically, all of these models are dynamic deterministic models, which means they pre-

dict how a system will unfold in time without any associated probability and spatial distribution (Thornley and Johnson 1990). Campbell and Madden (1990) distinguish between empirical models with a limited use for interpretation and mechanistic models, which give a greater opportunity to fulfil the three general modelling objectives: description, prediction and explanation of the behaviour of disease epidemics (Hau 1987). Empirical models provide a description of population growth based directly on the observation in a statistical sense with parameter estimations which are not biologically explicable. On the other hand, mechanistic models describe the performance of the population in terms of the processes known to underlie growth and development (Rimington and Charles-Edwards 1987). Mechanistic models have been classified as analytical and simulation models (Jeger 1986). The aim of analytical models is the description of the temporal course of epidemics, possibly using simple mathematical techniques such as one or few differential equations (Campbell and Madden 1990). Simulation models, on the other hand, try to describe the behaviour of complex systems. In this relationship, simulation models represent a tool of system analysis (Kranz and Hau 1980). While Jeger (1986) discussed the two approaches as contradictory, Kranz (1990) proposed the development of models from simple to complex ones and vice versa.

The application of the logistic model [Eq. (1)] to describe the temporal course of plant disease epidemics may be an example of a deterministic and mechanistic modelling technique. All parameters of this model [Eq. (1)] have explicable a biological meaning, the increase of disease  $dy$  will be determined from the interval of time  $dt$ , from the present amount of disease  $y$ , the disease free mass of the host  $1-y/K$  and the growth rate  $r$  i.e. rate of increase.

$$\frac{dy}{dt} = ry \left( 1 - \frac{y}{K} \right). \quad (1)$$

If one wishes to model the behaviour of two or more interacting populations, the Lotka-Volterra model (Lotka 1925; Volterra 1926) might be a promising basis. This model is based on logistic growth [Eq. (1)]. For two populations it consists of the following system of equations [Eq. (2)], where  $N_1$  and  $N_2$  are the number of individuals,  $r_1$  and  $r_2$  are the growth rates and  $K_1$  and  $K_2$  are the capacities which can be infected from the respective populations.  $N_1/K_1$  describes the influence of intraspecific competition of population  $N_1$  on the growth rate  $r_1$ , the same as in the logistic model [Eq. (1)]. The expression  $\alpha N_2/K_1$  indicates the influence of interspecific competition on the growth rate  $r_1$ .

$$\begin{aligned} \frac{dN_1}{dt} &= r_1 N_1 \left( 1 - \frac{N_1}{K_1} - \alpha \frac{N_2}{K_1} \right) \\ \frac{dN_2}{dt} &= r_2 N_2 \left( 1 - \frac{N_2}{K_2} - \beta \frac{N_1}{K_2} \right) \end{aligned} \quad (2)$$

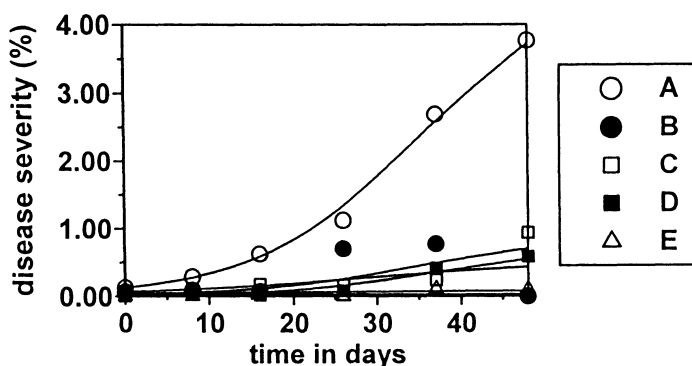


Fig. 6. Approximation of a system of linked differential equations from the Lotka-Volterra model to disease severity data of genotypes of *Erysiphe graminis* f. sp. *hordei*, differing in fungicide response to triadimenol and ethirimol. Successive samples were taken from an untreated barley field plot. Estimated growth rates are 0.103 for genotype A, 0.056 for B, 0.101 for C, 0.122 for D and 0.095 for E

If one approximates the differential equation of the logistic model [Eq. (1)] to disease severity data, the initial disease  $y_0$ , the rate of increase  $r$  and the capacity  $K$  can be estimated. Being more specific, disease is usually caused by a pathogen population, which is a composition of genotypes differing in some characters, e.g. virulence, fungicide sensitivity, RFLP or RAPD band patterns (see Sect. 3.a). The analysis of disease epidemics in this way is shown in Fig. 6, where each of the five genotypes (see Fig. 5) cause a portion of disease as a result of interaction. Approximation of a system of linked differential equations from the Lotka-Volterra model [Eq. (2)] to these data (Fig. 6) was carried out on the assumptions that the capacity is identical for all genotypes, the interspecific competition of the genotypes is 1, and the number of individuals  $N$  are equal to disease severity  $y$ . Now it is possible to estimate the initial disease  $y_0$ , and the rate of increase for  $r$  for each of the genotypes (see Fig. 6).

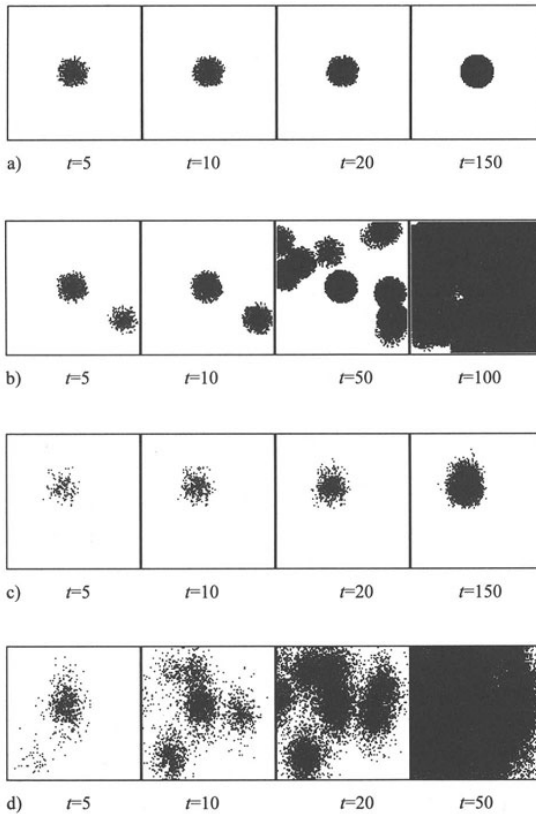
This example of deterministic modelling establishes a clear relationship between biological mechanisms and effects, meaning that each genotype will reproduce on a completely predictable basis at a constant rate. In reality, however, population growth is stochastic. One cannot assume that an individual will definitely reproduce in a specific time interval, but there is a certain probability of its doing so (Renshaw 1991).

The use of stochastic models in plant pathology has been reviewed by Shaw (1994). Moreover, the spatial distribution of the pathogen and the host might be relevant in plant disease epidemiology. Whether an individual plant is healthy or diseased depends often on the state of its neighbouring plants. A class of mathematical models which seem particularly appropriate to represent the spatial and stochastic nature of plant epidemics is based on cellular automata. These simulation models will be discussed in detail (see Sect. 4), here a single example is presented.



Constructing a spatially explicit model for the spread of diseases, the authors began with a single centre of infection on a two-dimensional grid, from which other cells could become infected at probabilities depending upon their distance from this centre [Eq. (3)]. The selected cumulative distribution function was:

$$\begin{aligned}
 p &= 1 && \text{if } s < 1 \\
 p &= 1 - \frac{a}{1-b} \cdot (s^{1-b} - 1) && \text{if } 1 \leq s \leq \left( \frac{1-b}{a} + 1 \right)^{\frac{1}{1-b}} \\
 p &= 0 && \text{if } s > \left( \frac{1-b}{a} + 1 \right)^{\frac{1}{1-b}},
 \end{aligned} \tag{3}$$



**Fig. 7.** Spatial patterns of the hypothetical spread of a disease after  $t$  time steps created by cellular automata with different rules based upon Eq. 3.  $a = 0.3$ ;  $b = 0.8$ . **a** Simple stochastic model; **b** with random emergence of new centres of infection; **c** simple model, but disturbed by wind, following a two-dimensional Gaussian distribution; **d** combined model with both emergence of new centres of infection and disturbance by wind

where  $s$  is the distance from the centre of infection and  $a > 0$  and  $0 < b < 1$  are parameters that determine the exact shape of the distribution. The distinction of three different cases of  $s$  is necessary to ensure that the probability does not exceed 1. The smallest  $s$  possible was 1, however. Note that there is a range, outside of which no infection will occur. During each time step every cell of the grid became newly infected with probability  $p$ .

Using the terminology that will be introduced in Section 4, this model is a cellular automaton with the three possible states 'not infected', 'infected' and 'centre of infection'. The neighbourhood set contains the whole grid, with weights according to the distance from the centres of infection. Running the model so far produces spatial patterns as shown in Fig. 7a. The model can be improved by allowing the emergence of new centres of infection. This was implemented by randomly creating new centres with probability  $p = 5 \cdot 10^{-4}$ . Adding this new rule changes the pattern of the cellular automaton quite drastically (Fig. 7b). The spread of a disease, however, will probably be affected by wind. Without consideration of elaborate existing climatic models, one easily can implement the effect of wind by assuming a two-dimensional Gaussian distribution (Campbell and Madden 1990). The rule is formulated as follows: If a new infection occurs, the location of the affected cell is moved  $x$  cells to the left and  $y$  cells upward.  $x$  and  $y$  are randomly drawn from normal distributions with mean  $m_x = 5$  and standard deviation  $s_x = 10$  and  $m_y = 5$  and  $s_y = 10$ , respectively. Starting with a single centre of infection, the model gives a more irregular pattern (Fig. 7c) than the one in Fig. 7a. Finally, the rules can be combined. Allowing for both the emergence of new infectious centres and for wind, the cellular automaton produces patterns like those in Fig. 7d.

This method of successively improving the rules of a cellular automaton is called 'top-down approach' (Kummer et al. 1994). The model results become more and more realistic as new rules are added.

#### 4. Spatially Explicit Models

An increasing interest in spatially explicit models has arisen over the past decade. On a large scale, spatially explicit models are used to model landscape dynamics and changes in land use or vegetation cover, while on a smaller scale single or several populations or organisms are of interest. Spatially explicit models are expected to increase our ability to accurately model populations subject to complex processes (Dunning et al. 1995; Kareiva and Wennergren 1995; Turner et al. 1995). Dynamic models of ecological communities should not neglect the spatial aspect; however, this is generally the case (Green 1989). Forecasting changes in populations can only be performed in a valid way by observing spatial dependences, because spatial relationships can radically change conditions for persistence and coexistence (Czárán and Bartha 1992). Bascompte and Solé (1995) noted that complex patterns may emerge from simple, spatio-temporal models. The complex structures of plant and pathogen distributions described in Section 3.c are possibly strongly affected by simple spatial interactions. Particularly useful spatially explicit models are cellular automata. Plotnick and Gardner (1993) refer to their use in epidemiological problems.

### a) Cellular Automata

Cellular automata are dynamic, spatially explicit models that are discrete in time, space and state. They consist of a number of cells or 'patches' (Dytham 1995), each of which can be in one of several possible states. The cells are connected and may vary in shape and connection pattern. Most models in the ecological literature consist of square cells, although some use hexagonal cells (e.g. Pulliam et al. 1992; Perry and Gonzalez-Andujar 1993). For reason of simplicity we will focus here on two spatial dimensions in the plane and on square cells. The way the cells are connected depends on their shape as well as on the range and geometry of connection. In the simplest case they are connected symmetrically according to a fixed spatial order. Only cells belonging to the defined neighbourhood of the cell, i.e. the cells connected with it, influence the state it will take after the next time step. Definitions of neighbourhoods often used for two-dimensional square grids are the *Moore-neighbourhood* consisting of the cell itself and eight neighbouring cells in directions N, NW, W, SW, S, SE, E and NE, and the *von Neumann-neighbourhood*, containing the cell itself and four cells in directions N, W, S and E. In a slight modification of Li (1995) the Moore-neighbourhood will be called second order and the von Neumann-neighbourhood will be called first order. Each cell can change its state during discrete time steps. The future state of each cell depends on its spatially neighbouring cells, according to specific transition rules which can be deterministic or stochastic (Czárán and Bartha 1992). An illustration of a simple cellular automaton with two possible states (0 and 1) is shown in Fig. 8. As time proceeds from  $t$  to  $t+1$ , each cell takes a new value depending on the states in its Moore-neighbourhood at time  $t$ .

Cellular automata appear in the literature under various other names, such as *individual-based dynamic automaton (IBDA)* model (Wiegand et al. 1994). Literature reviews concerning applications to ecology are given by Phipps (1992) and Balzter et al. (1996).

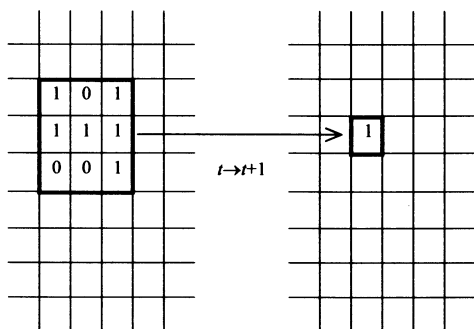


Fig. 8. Evolution of a 0/1 cellular automaton with the Moore-neighbourhood

If several rules are used, the order of processing these rules may be particularly important for the model results (Gilpin 1990; Ruxton 1996). Discussing the significance of cellular automata for ecological theory, Phipps (1992) concludes that, as far as applications to natural systems are concerned, stochastic rules usually have a better analogy to the system than deterministic ones, although their heuristic value is sometimes lower.

Cellular automata have a broad range of applications, for example modelling succession on resource gradients (Colasanti and Grime 1993), competition between plant species (Crawley and May 1987; Silvertown et al. 1992; Dytham 1995), distribution of genotypes of individuals (Epperson 1995), host-parasite interactions (Hassell et al. 1991), the impact of a power plant on a wetland community (Ellison and Bedford 1995) and vegetation dynamics (Van Tongeren and Prentice 1986; Green 1989; Marsula and Ratz 1994, Ratz 1994; Wiegand et al. 1994; Winkler et al. 1994; Loh and Hsieh 1995; Balzter et al. 1996).

Wolfram (1984) divided one-dimensional cellular automata into four qualitative classes, of which class three seems to be most common: Evolution leads to (1) a homogeneous state; (2) a set of separated simple stable or periodic structures; (3) a chaotic pattern; (4) complex localisation structures, sometimes long-lived. Packard and Wolfram (1985) found out that two-dimensional cellular automata may in fact be divided into the same four classes. On the other hand, Auger (1995) classified them into only three classes: static patterns, spiral waves and chaotic patterns.

A type of stochastic cellular automata commonly used is based upon an extension of the theory of Markov chains. In a Markov chain model, each individual can be in a discrete state (such as genotype or vegetation type). During discrete time steps the individuals can either keep their state or pass to another one with pre-assigned probabilities. The Markov assumption states that only the immediately preceding state influences the probabilities of transition to the next state. If the 'memory' of the Markov chain extends over two (or more) time steps into the past, this is called a second order (or higher) Markov chain. Models of this type are often used in biology. The Markov chain model lacks a spatial dimension, but it can be generalised by introducing the Markov property to cellular automaton models. All cellular automata governed by the simple stochastic rules outlined below are called *spatio-temporal Markov chain (STMC)* models. They possess a temporal order  $t$  as defined for simple Markov chains, and a spatial order  $n$  as mentioned for the cellular automata above. Conditions for STMC( $t,n$ ) models are:

1. The grid of cells must be symmetrical.
2. The transition rules must be purely stochastic, i.e. based on conditional probabilities.
3. There must be a spatial neighbourhood, on which the future of any cell depends. This neighbourhood can be restricted to only one cell,

resulting in an STMC( $t,0$ ) which is a simple, non-spatial Markov chain.

4. The future states of the cells must depend on the past states. The range of dependence determines the temporal order of the model. If the temporal order is 0, the model is an STMC( $0,n$ ), which is called a Markov random field (Guttorp 1995). In this case, the cell to be predicted is not allowed to belong to the neighbourhood. Finally, if both the spatial and the temporal orders are 0 [STMC( $0,0$ )], independent random events occur.

Two interesting applications of STMC( $t,n$ ) models were published by Silvertown et al. (1992) and Dytham (1995). Similar to Markov chains, the STMC( $t,n$ ) models tend in the long run towards a stable limiting distribution. Under certain circumstances this limiting distribution is independent of the initial distribution (called 'ergodic') and is determined solely by the transition probabilities. These properties allow some important conclusions about botanical phenomena to be drawn, to which models of that type can be applied successfully. For example, 'stable' plant communities following succession often show random fluctuations on a small scale, but are nevertheless stable under large scale observation. The STMC( $t,n$ )-type models probably belong to Wolfgram's class three; his class three examples appear to tend towards stable limiting distributions. The 'chaotic' behaviour causes continuous changes in states, but despite this the overall distribution of states may remain stable.

Great care must be taken when the structure of spatio-temporal dependence is defined. It was shown that neighbourhood definition affected the model results in a species-specific way. It could be that the correlation structure of the examined variables might give hints for selection of the right temporal and spatial orders of the model. Correlograms for different time and space lags may be useful for determining the right neighbourhood and the appropriate temporal dependence.

## b) Vegetation Dynamics

In an attempt to model population dynamics of three plant species on a lawn by a cellular automaton, several vegetation samples were taken from 1993 to 1995. The study site was located at the Agricultural Nursery in Giessen, Germany. Vegetation sampling was performed using the point-quadrat method, yielding spatial data for approximately 40 plant species. Study site and methodology are described in depth in Balzter et al. (1995). Population dynamics of three plant species were modelled. These are perennial ryegrass (*Lolium perenne*), a widely used forage grass which is also common in lawns and sports fields, white clover (*Trifolium repens*), belonging to *leguminosae* and also used as a forage

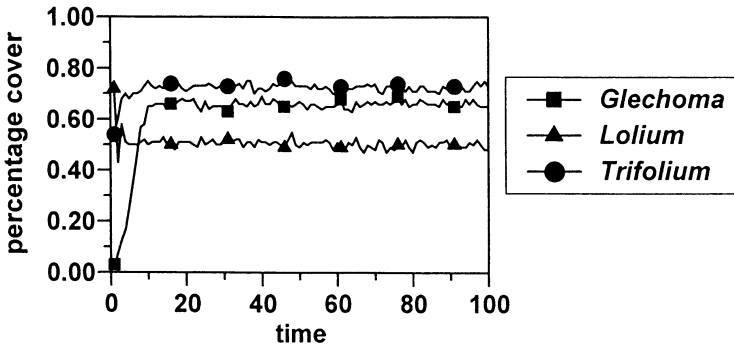


Fig. 9. Prediction of percentage cover over 100 time steps produced by cellular automaton using the Moore-neighbourhood model. (Means of ten simulation runs)

plant, and the ground ivy (*Glechoma hederacea*), a common ruderal species found usually on the edges of tracks but less frequently in the study area. All three species spread generatively and by above-ground stolons.

Of the several modelling approaches described in Balzter et al. (1996), only one is reported: a stochastic cellular automaton with the Moore-neighbourhood. Note that the STMC(1,2) model was called 'Moore-neighbourhood model' there. The data are spatially ordered in 12 columns and 10 rows, forming a total of 120 cells in the cellular automaton. Each cell can be either in state 1 (species present) or 0 (species absent). There are  $2^{12} = 512$  different combinations of cells belonging to the neighbourhood of a single cell. These were classified in order to provide accurate probability estimates. Transition matrices were estimated from the data by counting the numbers of transitions from any classified neighbourhood state into state 0 and 1, respectively (see Turner 1987 for further notes on transition matrix models).

Model results are shown in Fig. 9, showing ergodic distributions, i.e. the distribution of 0 and 1 converges independently from the initial configuration of cells to a stable distribution.

A problem arose at the edges with incomplete neighbourhood sets. In these cases the neighbouring cells were 'reflected' over the border and counted more times than usual. Reflection is considered more appropriate for vegetation data than other methods (see Haefner et al. 1991 for a comparison of methods), because the surroundings of the area are heterogeneous (a track, hedges of different type and cherry trees). If the number of cells increases, edge effects tend to vanish.

Model validation was performed using the data from June 1996, which is equivalent to the prediction at time step 1. The results are presented in Table 1.

**Table 1.** Percentage cover predicted for June 1996 by the STMC model vs. observed values

	<i>Lolium perenne</i> (%)	<i>Trifolium repens</i> (%)	<i>Glechoma hederacea</i> (%)
Predicted	51	70	17
Observed	5	16	16

Significant differences are observed for STMC vs. observations ( $p < 0.01$ ) for *Lolium perenne* and *Trifolium repens*, but homogeneity for *Glechoma hederacea* ( $p < 0.05$ ). Balzter et al. (1996) argued that the STMC model predictions for *Glechoma hederacea* are unlikely to match reality in the long-run, because the limiting distribution is higher than is expected from ecological intuition. However, surprisingly, the STMC model seems to produce reasonable output for a short time. The low observed values for *Trifolium repens* and *Lolium perenne* can be explained by an unexpectedly strong increase in rabbit population from 1993 to 1996. Here, we can formulate the hypothesis that due to an increase of the rabbit population, some plant species (e.g. forage plants such as *Lolium perenne* and *Trifolium repens*) (Spedding and Diekmahns 1972) are preferred food plants for these herbivores compared with others. Selective grazing can facilitate other populations, like *Glechoma hederacea*. This mechanism could cause changes in the transition matrices, which could then no longer be assumed to be stationary in time. The limiting distribution, and thus ecological equilibrium, is probably never reached in reality anyway, because in most ecosystems the environment is likely to change over longer time spans, thus influencing the organisms' dynamics and the underlying probabilities of the stochastic processes (Lippe et al. 1985). Successions in ecological communities seldom reach equilibrium or climax states. Instead communities tend towards a relative climax until environmental conditions change. For grazed communities the climax depending on the herbivore population is called the *zootic climax* (Daubenmire 1968).

A main problem of stochastic population models is the influence of unobserved (and often unobservable) variables that affect the probability structure as time progresses. Although proper planning of the sampling scheme can sometimes avoid this difficulty, exhaustive sampling of all relevant factors is often too expensive or not always possible for other reasons. In our study of the lawn dynamics in Giessen, the size of the rabbit population is suspected to be of particular importance. Although this was taken into account earlier as a possible cause for changing transition probabilities over time (Balzter et al. 1996), a longer time span than 3 years was considered to be necessary to influence vegetation dynamics that drastically.

Unobserved variables like these can be incorporated into the model in different ways: (1) The variable can be roughly estimated and taken as a covariate on an ordinal scale. This would result in the size values 'small', 'medium' and 'large' for the rabbit population in 1993, 1994 and 1995. The transition probabilities can be corrected according to a specified rule and the fit of the new model examined; (2) the stochastic processes can be made more complex by introducing unobserved states following themselves a Markov chain and affecting the states of vegetation. This new methodology of hidden Markov models was applied to precipitation

data by Zucchini and Guttorp (1991); (3) the influence of the unobserved variable can be determined in separate field trials under controlled conditions. Regression analysis or related methods quantify this influence deterministically, (4) the deviation of the prediction from observed reality can stimulate the generation of new hypotheses that could be tested in future studies.

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## Molecular Architects of Plant Body Plans

By Günter Theißen and Heinz Saedler

### 1. Introduction

#### a) What Are 'Molecular Architects of Body Plans'?

Generally, higher plants and animals generate offspring by sexual reproduction, implying that the adult individual is generated from a single fertilized egg cell, the zygote, during a complex process called development or ontogeny. One of the most fascinating questions of biology is how the diverse body plans of higher animals and plants, comprising tiny mosses as well as huge trees, small worms as well as giant whales and, yes, humans, are generated from very similar and simple beginnings, i.e. zygotes.

The progeny of mosses are always mosses, and that of worms are always worms, indicating that the body plan of the offspring is largely inherited from the parents. Clearly, plant and animal design has a genetic basis, but not all genes of the genome contribute to it in the same way. Some loci, carrying so called housekeeping genes, seem to be needed for basic cellular functions, such as transcription, translation, etc. However, some other genes, upon mutation, do not interfere with general cell functions (which would lead to embryonic lethality), but disrupt the spatial organization of the body by altering development. For example, some mutations in the *Antennapedia* gene of the fruit fly *Drosophila melanogaster* can cause the antennae on the head to be transformed into an extra pair of thoracic legs (McGinnis and Kuziora 1994). Similarly, some mutations in the *Deficiens* gene of the flowering plant snapdragon (*Antirrhinum majus*) cause the petals of the flower to be transformed into sepals and the stamens into carpels (Sommer et al. 1990). These kinds of mutations, where normal or wrong organs develop at a wrong place, are called homeotic (heterotopic). Other mutations, called heterochronic ones, alter the developmental timing and the duration of events. A third type, meristic mutations, changes the number of organs or suborgan components, e.g. the number of petals within a flower. As a consequence of yet another type of mutation, exemplified by



changes in gap genes of *Drosophila*, extended parts or segments of the body are missing.

That the malfunction of some genes affects the spatial organization of the body means that proper wild-type function of such genes is necessary for the correct formation of body design. Since mainly the body pattern is affected in some cases, it is reasonable to assume that it is the regular wild-type function of these genes to 'control', 'regulate', 'govern' or 'determine' pattern formation. In the following, the figurative term 'molecular architects' will be used for these genes with specific effects on pattern formation; thus this definition is a purely genetic one.

## b) Principles of Pattern Formation

Before we describe what has recently been learned about the genes that affect pattern formation in plants, we should keep in mind that pattern formation is just one out of four major developmental processes that generate the adult body in multicellular organisms. The three other processes are growth, morphogenesis and differentiation (Lyndon 1990). Growth e.g. by cell division, increases the size of the developing organism. Morphogenesis brings about the overall shape of the body by regional differences in orientation and rate of cell division, change of cell shape, and – in animals only – cell movement. Differentiation means the acquisition of cell type-specific features. Growth, morphogenesis and differentiation can thus readily be understood in terms of cell behaviour. In contrast, pattern formation refers to an abstract concept that addresses the organizational aspect of development: the different cells, tissues and organs that make up the adult body have to originate at, or move to, specific locations in order to form a meaningful context, i.e. pattern. Thus, pattern formation generates the basic features of the body organization, whereas morphogenetic activities, such as localized cell divisions or changes in cell shape, generate the body structure.

Pattern formation can be understood as a two-step process (Wolpert 1996). First, cells are informed of their position in a three-dimensional coordinate system, for example, by gradients of morphogens. Second, they interpret this information to form the appropriate structures by location-specific morphogenetic, differentiation and growth processes. Morphogenesis and pattern formation, as defined here, are often mixed up, or morphogenesis is considered as comprising pattern formation. However, both processes can be uncoupled genetically.

For example, *fass* mutant seedlings of the model flowering plant *Arabidopsis thaliana* are stout, short and compressed in the apical-basal axis, but all pattern elements of the seedling are present. *Fass* mutant cells are round and irregularly spaced, whereas the cells of the wild-type seedling are elongated and stacked. Thus, morphogenesis is highly dis-

turbed in *fass* mutants, resulting in an abnormally shaped seedling, whereas pattern formation is largely normal (Torres Ruiz and Jürgens 1994).

Accordingly, morphogenesis and pattern formation should be properly distinguished. Here we pay special attention to genes that affect pattern formation (i.e. the formation of the 'body plan'), but, where appropriate, some genes affecting morphogenesis (i.e. the formation of the 'body structure') are also considered briefly.

### c) Plant Versus Animal Development: Differences and Peculiarities

During the last 20 years we have learned a great deal about pattern formation in animals. In one animal model system, *Drosophila melanogaster*, we have already a relatively detailed and thorough understanding of how the basic body pattern is laid down during embryogenesis (Lawrence 1992). However, plants have body plans that are quite different from those of animals. There are several reasons for this. The most important one is that multicellular plants and animals have independent phylogenetic origins, because they evolved independently from unicellular ancestors (Chasan and Walbot 1993). Therefore, animals and plants share general eukaryotic features (e.g. special characteristics of the basic cell metabolism, the transcription and translation apparatus, etc.), but the body plans of animals and plants are truly non-homologous – they are independent 'inventions' of nature. In order to recognize general trends in the phylogeny of body plans (macroevolution), it is therefore highly instructive to compare the evolution of plant body plans with that of animal body plans. Since the body plans of multicellular organisms are generated by complex developmental processes, one may assume that animal and plants have established different developmental strategies throughout their independent evolution. That is indeed the case.

For example, while the basic body plan of animals is already established in the embryo or larval stages, plants have a constant proliferation of new organs beyond embryogenesis. They are thus able to form new elements of their body pattern (e.g. leaves or flowers) according to environmental requirements. The postembryonic organ formation of plants is initiated from undifferentiated proliferative zones called meristems. As cells leave the meristem regions, cell identity is determined almost entirely by the position of one cell relative to its neighbours, rather than by its genealogy, suggesting that cell-cell interactions play an important role in the establishment of plant cell fate. In contrast, cell lineage, i.e. the genealogy of cells, plays the major role in determining the identity of animal cells. That cell position is of relatively higher importance during plant than during animal development is also due to the fact that plant cells cannot move because of their rigid cell wall. Therefore, in contrast

to animal systems, cell movement plays no role during plant development. The final size and shape of plant organs thus record the history of past cell divisions.

Another major difference in the developmental strategies of plants and animals is that animals contain a germ line, whereas the gametes of plants differentiate late in development from a population of cells that have been actively engaged in organizing the somatic body of the plant. Mutant cell lineages of plants are thus 'tested' somatically before they give rise to germ cells. Therefore, somatic tissue of plants must be able to reprogram into reproductive tissue. In line with this, plant cells are often totipotent, in contrast to animal cells. Furthermore, the life cycle of plants includes two different phases, a haploid gametophyte which produces the gametes, and a diploid sporophyte, which contains cells that can undergo meiosis. In contrast, most animals are diploid species, with only their gametes being haploid.

The differences in the developmental strategies of plants and animals reflect the fact that natural selection favoured very different life strategies in case of these two organism lineages (Chasan and Walbot 1993). Due to the fortunate acquisition of a prokaryotic, photoautotrophic endosymbiont during evolution, plants have generally an autotrophic lifestyle that depends on efficiently absorbing sunlight. On the contrary, animals are heterotrophic organisms that depend on finding organic food. Therefore, it proved highly advantageous for most animals to move, while plants evolved as immotile organisms. (The existence of higher fungi, however, indicates that being heterotrophic, yet sessile is also a reasonable way of living for multicellular organisms.) The behaviour and physiology of an animal is thus adapted to the fact that it can flee if it encounters a threat, and that it can actively search for what it is lacking (e.g. food, water, mating partners). In contrast, plants have to cope with stress and demands at the location where they are, meaning that they have to do so by developmental or physiological strategies.

Since cell movement plays no role during plant development, the body shape and patterns of plants largely document the rate and orientation of cell division and cell elongation. Accordingly, the number and planes of cell divisions and the orientation and dimensions of cell elongation are under tight control in regular plant development (for a review, see Meyerowitz 1996). The existence of mutants like *fass* (see Sect. 1.b), however, strongly suggests that cell shape is not very relevant for the formation of the basic body pattern of the seedling. Similarly, it turned out that the generation of maize (*Zea mays*) leaf shape does not depend on the precise spatial control of cell division. The maize *tangled1* mutation, for example, causes cells to divide in abnormal orientations throughout leaf development without altering overall leaf shape (Smith et al. 1996). Even more surprising was the recent finding that gross changes in the rate of cell division had little or no influence on the for-

mation of the plant body pattern (for a review, see Doonan and Hunt 1996).

For example, by overexpressing B-cyclin in *Arabidopsis*, Doerner et al. (1996) increased the number of cells in the roots of transgenic plants. However, although the roots were longer, they otherwise looked entirely normal, demonstrating that the transgenic plants incorporated the extra cells into their normal body plan. A similar tolerance of plants with regard to the number of cells that they require for the formation of their body plan was observed in a complementary experiment, where cell number was decreased. Hemery et al. (1995) made transgenic *Arabidopsis* plants expressing an inactive cyclin-dependent kinase (CDK) protein which forms non-productive complexes with cyclins. Surprisingly, these transgenic plants grew at almost the same rate and to approximately the same size, although they contained less than half the normal number of cells. Thus, the decrease in cell number is compensated in each plant organ by a concomitant increase in the size of individual cells.

From these results it can be concluded that the formation of the plant body pattern is not simply controlled by a program that determines the rate and orientation of cell division and cell elongation. The genes controlling the different parameters of cell division are thus not the 'molecular architects of plant body plans'. How is this possible, given that the rate and orientation of cell division and cell elongation, and almost nothing else, ultimately determine plant shape and pattern? A possible answer might be that plant global development is subject to local control (Meyerowitz 1996): plant cells may be informed if and how their neighbours are dividing, and they may use this 'knowledge' to decide whether to divide or not, and to 'choose' their plane of cell division. Cell-to-cell communication, e.g. by the selective trafficking through plasmodesmata of mRNAs or proteins encoded by some developmental 'control' genes (see Davies 1996, and references therein), may play pivotal roles in the local control of cell behaviour. In any case, mechanisms different from the control of cell division number and orientation must be involved in the generation of pattern and shape during plant development.

Several interesting questions arise: if there is a local control of cell division in plants, how then is the proper formation of the global plant body pattern 'governed'? What kind of genes are 'molecular architects of plant body plans'? Are they a homogeneous class of genes? What is their function in biochemical terms? Are they principally different from those of animals, given that multicellularity of animals and plants is non-homologous? Do they interact and thus constitute a 'program' that 'governs' development, in analogy to neural or computational networks that 'control' animal behaviour or computational processes?

To answer these questions, as far as is possible today, let us follow the life cycle of a plant and see what has recently been learned about the 'molecular architects of plant body plans'.

Due to space constraints only selected aspects of the topic are reviewed here, and the authors apologize for not citing all of the relevant papers of their colleagues. 'Molecular' means that the text will focus on genes that have already been cloned, facilitating insights into their molecular mode of action. Only publications from the 1990s are cited, preferably from the last 2 years. Rather than presenting complete lists of mutants or genes, interesting case examples are discussed and specialized articles are referred to for more comprehensive reviews of the different subtopics. The text concentrates on *Arabidopsis* and maize as well advanced dicotyledonous and monocotyledonous model systems, respectively, and mentions genes from other species only in exceptional cases. For brevity, the terms 'plant(s)' or 'animal(s)' are often used where 'vascular plant(s)', 'seed plant(s)', 'flowering plant(s)' or 'bilaterian animal(s)', respectively, would be more accurate terms.

## 2. Genes Controlling Pattern Formation

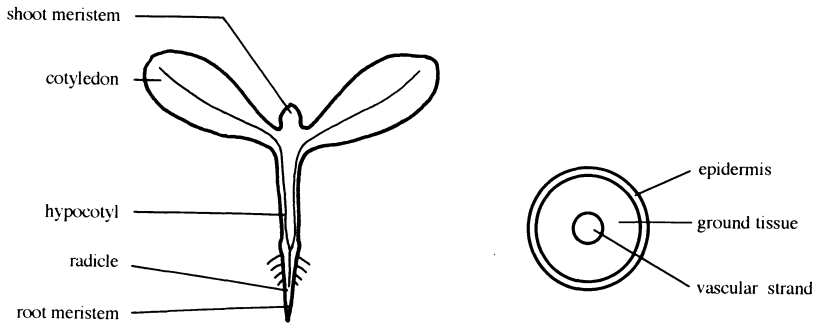
### a) Development of the Sporophyte

#### α) Embryogenesis

The ontogeny of the sporophyte starts with the union of two haploid gametes in a diploid zygote. The following development stages, from the one-cell zygote to the mature embryo, i.e. embryogenesis, have been well characterized at the morphological level in *Arabidopsis* (Jürgens et al. 1991).

The *Arabidopsis* zygote divides asymmetrically to generate a smaller apical cell, which gives rise to the embryo proper, and a larger basal cell from which the quiescent centre of the root meristem, the central portion of the root cap and the suspensor arise. The two-cell embryo undergoes precise cell divisions and develops through a globular stage, a heart stage (where bilateral symmetry is established), a torpedo stage and a curled cotyledon stage until it becomes mature.

Eventually, the mature embryo will germinate to produce a seedling, a juvenile plant of stereotyped appearance. The seedling has a simple body organization, which can be described as the superimposition of two patterns, one axial (apical-basal) and one radial (Fig. 1; Mayer et al. 1991; Jürgens et al. 1994; Jürgens 1995). The apical-basal pattern is arranged along the single axis of polarity. The following pattern elements are found in the seedling (from apical to basal): a shoot meristem, cotyledons (embryonic leaves), a hypocotyl (embryonic stem) and a radicle (embryonic root) including a root meristem. The root and shoot meristems generate the adult structures (leaves, flowers, roots, etc.) during postembryonic development (see Sect. 2.a.β,γ). The radial pattern involves three major tissues of the primary plant body: the outer epidermis, the inner mass of ground tissue (cortex and endodermis), and the centrally located vascular strands (pericycle, xylem and phloem). Each of the apical-basal pattern elements consists of, or gives rise to, all three



**Fig. 1.** Pattern elements (body plan) of the seedling. *Left* Seedling habitus, revealing apical-basal pattern; *right* schematic cross-section through hypocotyl region, revealing radial pattern. [This figure is based on a concept outlined by Mayer et al. (1991)]

tissues (Mayer et al. 1991). Morphological evidence suggests that during embryogenesis the basic body pattern has already been laid down by the heart stage (Jürgens et al. 1991; Mayer et al. 1991). Later embryogenesis predominantly involves further growth of the primordia by cell division and the differentiation of cells.

How can the genes that 'control' pattern formation during plant embryogenesis be identified? Since the authors' definition of these genes is a purely genetic one (Sect. 1.a), the methodology of genetics has to be applied, of course. Plant geneticists disrupt biological processes by making (or just looking for) mutations and examining the consequences in the respective mutant plants. That is exactly what Jürgens and his coworkers did in a pioneering study that was reported half a decade ago (Mayer et al. 1991). They had carried out a systematic search for mutations in *Arabidopsis* that disrupt the spatial organization of the seedling by altering embryogenesis (Mayer et al. 1991). In their screen, they pre-selected putative pattern mutants on the basis of altered body organization of the seedling, in order to eliminate mutants defective in more general cell functions. Mayer et al. found mutations in six genes that affect both aspects of body pattern: the apical-basal pattern and the radial pattern. Mutations in four genes, *GURKE*, *FACKEL*, *MONOPTEROS*, and *GNOM*, delete large regions of the apical-basal pattern without affecting the formation of the primary body tissues. *gurke* mutants lack the apical region of the seedling, i.e. cotyledons and the shoot apical meristem; *fackel* mutants have the cotyledons attached to the root, indicating that they lack the central region (hypocotyl); *monopteros* mutants lack the hypocotyl and the root, i.e. the basal region of the seedling; *gnom* mutants lack both apical and basal elements, resulting in seedlings that are cone- or ball-shaped.

The mutant phenotypes of *gurke*, *fackel*, *monopteros* and *gnom* suggest that apical-basal pattern formation involves partitioning of the axis into three major regions: apical, central and basal. This partition of the embryo occurs at a very early stage, probably in response to positional information derived from the polarized egg cell. The partitioning may be affected by the four patterning genes in either of two ways (Mayer et al. 1991). As suggested by the pairs of complementary mutant phenotypes, the genes could act in a combination mode (similar to the genes specifying floral organ identity; see Sect. 2.a.γ): *GURKE* and *GNOM* would specify apical, *FACKEL* and *MONOPTEROS* central, and *GNOM* and *MONOPTEROS* basal. Alternatively, the four genes may interact in some hierarchical fashion.

Mutations in two other genes, *KNOLLE* and *KEULE*, seem to affect the radial pattern of the seedlings.

Most *knolle* seedlings look round or tuber-shaped. In *knolle* embryos, one cannot morphologically distinguish an outer layer of epidermal precursor cells from an inner cell group. Rather, the early embryo seems to consist of enlarged cells which are irregularly spaced. In addition, the upper part of the suspensor is enlarged as if this region had become part of the embryo. Mutations in *KEULE* cause defects in the epidermis, although the epidermal precursor cells form in *keule* embryos. However, they are abnormal from the very beginning. In the resulting *keule* seedlings, the cells of the ground tissue and the vascular strands look normal, whereas the epidermal cells are bloated and irregularly arranged. The seedlings shape varies, often approaching an elongated structure topped with strongly reduced cotyledons.

Interestingly, the epidermis is the only affected pattern element in both radial pattern mutants, and Mayer et al. (1991) failed to isolate mutations affecting the other two tissues. As possible explanations the authors suggested that either the other tissues can be replaced by re-specification of adjacent tissue or drastic alterations of the radial pattern interfere with seed germination (such mutants would have been missed in Mayer et al.'s screen).

In addition to the pattern mutants, Mayer et al. (1991) described also three shape mutants, *fass* (already mentioned in Sect. 1.b), *knopf* and *mickey*, that are defined by grossly abnormal seedlings which, however, retain all the pattern elements found in wild-type seedlings. Thus, morphogenesis, but not pattern formation, is disturbed in these three mutants.

Since their first description, some of the pattern mutants have been characterized in more detail (Berleth and Jürgens 1993; Mayer et al. 1993, Assaad et al. 1996; Przemeck et al. 1996). Some of these studies provided evidence that the function of the analysed genes is not restricted to embryogenesis, but rather is needed at multiple stages throughout the plant life cycle.

Recently, some additional insights into the pattern defects could be obtained by using the expression of the lipid transfer protein gene *ATLTP1* as a tissue-specific molecular marker in mutant embryo analysis (Vroemen et al. 1996). In wild-type embryos, the *ATLTP1* gene is initially expressed in all protoderm cells (the precursors of the epidermal

cells); later, *ATLTP1* expression is confined to the cotyledons and upper end of the hypocotyl. Analysis of *ATLTP1* expression in *gnom*, *knolle* and *keule* embryos demonstrated that *gnom* embryos can have no or reversed apical-basal polarity, whereas the radial pattern is unaffected, *knolle* embryos initially lack but later form a radial pattern, whereas *keule* embryos are affected in protoderm cell morphology rather than the establishment of the radial pattern (Vroemen et al. 1996).

Two of the genes that are affected in the pattern mutants, *GNOM* and *KNOLLE*, have been cloned up to date, so that the molecular characterization of these genes could be initiated. The *GNOM* gene (also called *EMB30*) was isolated independently in two different laboratories, employing T-DNA tagging (Shevell et al. 1994) or positional cloning (Busch et al. 1996). *GNOM* encodes a 163-kDa protein that has a conserved domain in common with the yeast secretory protein *SEC7* (Shevell et al. 1994). However, analysis of *gnom* mutants by electron microscopy did not reveal any defects in the Golgi apparatus similar to those observed in the yeast *sec7* mutants (Shevell et al. 1994; Busch et al. 1996). Furthermore, the translation product of a different yeast gene, termed *YEC2*, is more similar than the *SEC7* protein to the *GNOM* protein in size, overall sequence, and number and distribution of conserved sequences, suggesting that *YEC2* is a better candidate for a *GNOM* orthologue in yeast than *SEC7* (Busch et al. 1996). Deletion of the *YEC2* gene by homologous recombination did not affect growth or cell division, indicating that this gene, like *GNOM* itself, is not required for cell viability (Busch et al. 1996). The *GNOM* gene is constitutively expressed during development (Shevell et al. 1994), and none of the regions conserved between *GNOM* and the *YEC2* protein (including the *SEC7* domain) has been characterized functionally. Therefore, neither the *GNOM* sequence nor its expression pattern give specific clues to its function during embryogenesis. Thus, we are in a situation not uncommon in molecular biology today: a gene has been cloned and sequenced in its wild-type and several mutant states, its mutant phenotype has been well characterized, and some remarkable genetic phenomena have been observed (like intragenic complementation, suggesting that *GNOM* works as a homomeric multimer; see Busch et al. 1996). However, what the *GNOM* protein is actually doing at the molecular level in the cell is absolutely unknown so far. The identification of other factors interacting with *GNOM* may help to clarify that issue in the future.

The *KNOLLE* gene was also isolated by positional cloning (Lukowitz et al. 1996). It encodes a predicted 34-kDa protein with similarity to syntaxins, a family of proteins involved in vesicular trafficking. Syntaxins are thought to be target membrane receptors that interact with corresponding vesicle receptors and soluble factors to promote the fusion of target and vesicle membranes. At the cellular level, *knolle* embryos are characterized by incomplete cross walls and enlarged cells with polyploid nuclei, indicating that cytokinesis is affected in these embryos.



Cytokinesis is the process that partitions the cytoplasm of a dividing cell following the separation of the daughter chromosomes. Since the transport of vesicles to, or their fusion at, the plane of cell division is an important aspect of cytokinesis, it could well be that *KNOLLE* has syntaxin-like functions during cytokinesis. *KNOLLE* expression was observed throughout the plant, but only single cells or small groups of adjacent cells accumulate *KNOLLE* RNA, consistent with the notion that *KNOLLE* transcripts are produced only during a specific phase of the cell cycle (Lukowitz et al. 1996). According to recent studies based on light and electron microscopy the *KEULE* gene is also involved in cytokinesis (Assaad et al. 1996).

For many genes that are expressed during embryogenesis no function or mutant phenotype has been established so far. Among these are two types of genes that seem remarkable to us, because they belong to gene families which comprise also members that have well-known functions in developmental 'control' of non-embryogenic processes.

The first example is *AGL15*, a member of the MADS-box gene family [*MCM1* (from *Saccharomyces cerevisiae*), *AGAMOUS* (from *A. thaliana*), *DEFICIENS* (from *A. majus*) and *SRF* (from *Homo sapiens*)]. All MADS-domain proteins of known function are transcription factors which regulate the expression of target genes by binding to specific *cis*-acting DNA sequences. They share a sequence element of about 60 amino acids, the MADS-domain (Schwarz-Sommer et al. 1990), which functions in DNA binding and protein-protein interactions and is highly conserved across developmental control genes from yeast to animals and plants (Shore and Sharrocks 1995; Theißen and Saedler 1995; Theißen et al. 1996).

MADS-domain proteins have a fascinating range of biological functions. They are involved in controlling processes as different as arginine metabolism and mating type determination (in yeast), growth factor response and muscle development (in vertebrates and insects), trachea development (in insects), and inflorescence and flower development (in higher plants). For example, the majority of floral meristem and organ identity genes are MADS-box genes (for recent reviews, see Ma 1994; Meyerowitz 1994; Weigel and Meyerowitz 1994; Theißen and Saedler 1995; Theißen et al. 1996; see Sect. 2.a.γ). The expression patterns of other MADS-box genes suggest that they might be involved in the control of fruit development, vegetative growth or root development (for recent reviews, see Theißen and Saedler 1995; Theißen et al. 1996).

*AGL15*-like genes constitute a new subfamily within the multigene family of MADS-box genes (Theißen et al. 1996). Although cDNA and genomic clones of *AGL15*-like genes have been reported only from Brassicaceae species up till now (Heck et al. 1995; Rounsley et al. 1995), this type of gene is probably present in most, if not all angiosperms (Heck et al. 1995; Perry et al. 1996). In *Brassica napus*, *AGL15* mRNA accumulates primarily in the embryo and is present in all embryonic tissues, beginning at least as early as late globular stage (Heck et al.

1995). In *Arabidopsis*, a low level of expression was found in rosette leaves (Rounsley et al. 1995). Given the prominent functions of some of its gene family members, it would not come as a surprise if *AGL15* plays an important role in the developmental 'control' of embryogenesis.

The second example is some members of another 'prominent' gene family encoding transcription factors involved in developmental 'control', the homeobox genes (Gehring et al. 1994). For example, all *HOX* genes – these genes have been called the 'molecular architects of (animal) body design' (McGinnis and Kuziora 1994) – are members of the homeobox gene family. Like the MADS-domain, the homeodomain is also a sequence element of about 60 amino acids which functions in DNA binding, and it is also highly conserved across developmental control genes from yeast to animals and plants (Gehring et al. 1994). The study of some plant homeobox genes, like *KNOTTED1* and *GLABRA2*, provided evidence that these genes contribute to developmental decisions or have features reminiscent of selector genes operating during *Drosophila* development (see Sect. 2.a.β; for a review, see Gasser 1996). It is remarkable, therefore, that some plant homeobox genes are expressed during embryogenesis. For example, expression of the *ZMHOX* genes from maize is activated very early in embryonic development and restricted to the embryo proper in the proembryo stage (Klinge and Werr 1995).

After establishment of the root/shoot axis, transcripts are prevalent in the embryonic root and shoot apical meristems, but later are also present in provascular tissues and young leaf primordia. However, expression of *ZMHOX* genes is also found in the postembryonic plant body. Four different genes have been isolated till now from maize, called *ZMHOX1a*, *1b*, *2a* and *2b*, but some more *ZMHOX* genes are very likely present in the maize genome (Klinge et al. 1996).

The rice (*Oryza sativa*) homeobox gene *OSH1* is first expressed at the globular stage of embryogenesis, much earlier than organogenesis is started, in a ventral region where shoot apical meristem and epiblast would later develop, indicating that cellular differentiation has already occurred at this stage (Sato et al. 1996). At later stages, *OSH1* expression was observed in shoot apical meristem, epiblast, radicle and their intervening tissues. Interestingly, in an embryo mutant that develops no embryonic organs, *OSH1* expression is the same as that in the wild-type embryo, demonstrating that *OSH1* is not directly associated with organ differentiation. The *OSH1* expression pattern during embryogenesis suggests that, like animal homeobox genes, *OSH1* plays an important role in regionalization of cell identity during early embryogenesis (Sato et al. 1996).

Taken together, although there is no direct proof to date that homeobox genes are important for a proper formation of plant embryo

architecture, manifold circumstantial evidence suggests that this is indeed the case.

### β) Vegetative Development

Plant embryos and seedlings contain only a small fraction of the final body plan (see above). The adult plant structure is established during postembryonic development by the activity of the root and shoot apical meristems, two groups of proliferative cells.

### Root Development

The initial cells forming the root are provided by the root apical meristem near the root tip.

Cell division at the distal edge of the root meristem produces the cells of the root cap, and proximal cell divisions add to a zone of cell proliferation next to the meristem. In this proliferation zone, the number of root cells is substantially increased by cell divisions. At the proximal edge of this region cells enter an elongation zone in which most of the increase in root length occurs. The elongated cells then enter a differentiation zone in which the functional specializations of the different cells become apparent. Branch roots appear that define root modules analogous to the phytomers of the shoot. They generally originate from the pericycle, a cylindrical tissue surrounding the central core of vascular tissue in the root. The epidermis of the wild-type root is composed of two different cell types, root-hair cells and hairless cells. They are located at distinct positions within the root, suggesting that positional cues control cell-type differentiation (for a review, see Dolan and Roberts 1995).

A number of genes are known to be involved in postembryonic root pattern formation (for a review, see Scheres et al. 1996). For example, the *SCARECROW* gene of *Arabidopsis* regulates an asymmetric cell division that is essential for generating the radial organization of the root (Di Laurenzio et al. 1996). In *scarecrow* mutants, asymmetric periclinal divisions of the cortex/endodermal initial are disrupted, resulting in the loss of a cell layer between the epidermis and pericycle of the root. A similar mutant phenotype is caused by mutations at the *SHORTROOT* locus. Recently, the *SCARECROW* gene could be cloned by T-DNA tagging (Di Laurenzio et al. 1996). It encodes a gene product that has some motifs – such as a region similar to the basic domain of typical basic-leucine zipper (bZIP) proteins – that strongly suggest that the *SCARECROW* protein acts as a transcription factor.

Some mutants, such as *glabra2*, *transparent testa glabra* and *constitutive triple response1*, display an altered distribution of hair and non-hair files in the root epidermis (e.g. ectopic hair formation). Two of these genes have been cloned and were shown to encode a member of

the Raf family of protein kinases (*CONSTITUTIVE TRIPLE RESPONSE1*; Kieber et al. 1993) or a homeodomain protein (*GLABRA2*; Rerie et al. 1994), respectively. Recent studies demonstrated that *GLABRA2* acts in a cell-position-dependent manner to suppress hair formation in differentiating hairless cells (Masucci et al. 1996). Based on genetic, molecular and physiological tests, Masucci and Schiefelbein (1996) have developed a model for the control of root epidermis development. The model describes the interaction of the three genes mentioned above with two other genes, *ROOT HAIR DEFECTIVE6* and *AUXIN RESISTANT2*, and the plant hormones ethylene and auxin in root hair formation.

Interestingly, some mutants are known which have defects only in secondary roots, suggesting that primary and lateral root primordium formation have different genetic requirements. The *alf4* mutant of *Arabidopsis*, for example, specifically lacks detectable lateral and adventitious root primordia (Celenza et al. 1995); thus, the *ALF4* gene may be involved in the spatial definition of the lateral root founder cells as a patterning gene. Similarly, *rtcs* mutants of maize are characterized by a complete lack of formation of crown and lateral seminal roots; they survive by the ability of the primary root to support the growth of the developing plant (Hetz et al. 1996). That primary and lateral root formation can be uncoupled genetically fits well to the differences in primary and secondary root primordium formation. For example, in the *Arabidopsis* embryo, the root is specified as an element of the apical-basal embryonic pattern, depending on the activity of patterning genes such as *MONOPTEROS* (see Sect. 2.a.α). On the contrary, to initiate *Arabidopsis* lateral root formation, pericycle cells need to dedifferentiate and divide, a process which is perhaps controlled by *ALF4*.

## Shoot Development

The shoot structures of flowering plants are generated by shoot apical meristems, small groups of undetermined cells located at the distal-most portions of the shoots. These cells are the source of literally all the cells that compose the above-ground portion of the plant. The shoot apical meristem initiates tissues and organs, communicates with other parts of the plant and maintains itself as a formative region. It is the regulation of these functions in a temporal and spatial manner that generates a shoot with a predictable patterned form.

The developmental pattern generated by the vegetative shoot apical meristem is segmental. Therefore, the architecture (body plan) of the shoot is composed of reiterative units termed phytomers. Each phytomer consists of a leaf, leaf node, internode (stem segment) and axillary bud. Histological studies and clonal analyses have shown that in maize a leaf belongs to the internode and axillary bud below it, though this is not the case in all plants (Jackson et al. 1994, and references therein).

The shoot apical meristem produces sequentially new phytomers, whose morphology may change over the course of the plant's development (for a review, see Lawson and Poethig 1995). Variations of segment

structure arise by variation of the internodal length, suppression of leaf or branch development, or transformation of leaves or branches into specialized structures.

The *teosinte branched1* mutant of cultivated maize (*Zea mays* ssp. *mays*), for example, has elongated lateral branches that are terminated by male inflorescences, so that the mutant looks like a candelabra. The putative wild ancestor of maize, the Mexican grass teosinte (*Zea mays* ssp. *parviglumis*), has a very similar growth form (Doebley 1992; Saedler and Theißen 1994, Doebley et al. 1995). In wild-type maize, the lateral branches are short and terminated by female inflorescences. Changes at the *teosinte branched1* locus of teosinte were very likely involved in the dramatic change in plant architecture during maize domestication (Doebley et al. 1995). The transition from teosinte to maize, which took only a few thousand years of domestication or even less, and the *teosinte branched1* mutant of maize are thus two perfect case examples demonstrating that a mutation at a single locus can dramatically change the plant habit (Doebley 1992, Saedler and Theißen 1994, Doebley et al. 1995).

In *Arabidopsis* there are also several mutations known that drastically change the vegetative body plan.

For example, in *revoluta* plants, axillary meristems frequently fail to develop in the axils of rosette and cauline leaves, resulting in branchless plants (Talbert et al. 1995). Plants homozygous for the *embryonic flower* mutation lack the basal rosette of leaves and germinate into leaf- and flower-bearing stems directly (Yang et al. 1995, and references therein). On the contrary, some *Arabidopsis* mutants and ecotypes that are delayed in the transition to flowering have an increase in the number of rosette leaves (for a review, see Haughn et al. 1995).

Recently, Grbic and Bleeker (1996) reported an interesting late-flowering ecotype, termed Sy-O, in which the axillary meristems maintain a prolonged vegetative phase, even though the primary shoot apical meristem has already converted to reproductive development. This heterochronic shift in the development of axillary meristems results in the formation of aerial rosettes of leaves at the nodes of the primary shoot axis. The remarkable change in the *Arabidopsis* body plan arises due to the interaction between dominant alleles of only two genes, termed AERIAL ROSETTE GENE (*ART*; on chromosome 5) and ENHANCER OF AERIAL ROSETTE (*EAR*; on chromosome 4). *EAR* may be a new allele of the *FRIGIDA* locus (Grbic and Bleeker 1996).

Another interesting vegetative and reproductive syndrome, including multiple rosettes, stem fasciation, retarded senescence, delayed flowering, determinate inflorescences, etc., is caused by changes of the *WALDMEISTER* locus on chromosome 1 (Felix et al. 1996).

A key feature of vegetative plant growth that strongly determines the vegetative plant form above ground is the arrangement of leaves around the axis of growth, i.e. phyllotaxy.

Leaves can be initiated singly, separated by 180° (an arrangement called distichous) or in a spiral pattern (e.g. the rosette leaves of *Arabidopsis*). Whorled patterns result from the simultaneous initiation of two or more leaves. An example is the decussate whorled arrangement of the vegetative *Antirrhinum* shoot, where an opposite pair of leaves is initiated simultaneously that is offset by 90° from the previous pair.

A series of *Arabidopsis* genes is known that, upon mutation, result in alterations in the positions where leaf primordia form (i.e. phyllotaxy), such as *CLAVATA1*, *CLAVATA3*, *FASCIATA1*, *FASCIATA2* and *FOREVER YOUNG* (Leyser and Furner 1992; Callos et al. 1994; Clark et al. 1996). The *CLAVATA* and *FASCIATA* mutant phenotypes are associated with enlarged and morphologically abnormal shoot apical meristems, illustrating a close relationship between the geometry of the shoot apex and leaf initiation patterns. A similar phenotype in maize is known as the 'ABPHYL syndrome', where the normally distichous phyllotactic pattern is changed to a decussate or spiral pattern (reviewed by Smith and Hake 1992). This syndrome is also associated with an increase in apical meristem size. The corresponding genes seem to exert their function by affecting meristem size, possibly by negatively regulating proliferation in the meristem. The *FOREVER YOUNG* gene could be cloned and was shown to encode a protein with high similarity to a nodulin (a class of plant proteins expressed in nitrogen-fixing root nodules) and limited similarity to various reductases. It has been proposed that the *FOREVER YOUNG* protein plays a role in communication in the shoot apex through the modification of a factor regulating meristem development (Callos et al. 1994).

How the different components of the phytomer become organized from initially indeterminate cells in the shoot apical meristem is still largely unknown. It is becoming more and more clear, however, that some homeobox genes play important roles in maintaining the indeterminacy of the meristem. One of the most important results in that respect during recent years was the cloning of the *Arabidopsis* gene *SHOOTMERISTEMLESS* (*STM*) (Long et al. 1996; for an overview, see Hake 1996). *Arabidopsis* seedlings in which *STM* is defect have functional root meristems and cotyledons, but no leaves. From the analysis of the *STM* loss-of-function phenotype it had been concluded that this gene is required for the shoot apical meristem to form (Barton and Poethig 1993). Using the homeobox of the maize gene *KNOTTED1* (see below) as a hybridization probe, a cDNA of an *Arabidopsis* gene homologue was isolated that mapped very close to the *STM* locus on *Arabidopsis* chromosome 1. Additional experiments confirmed that the isolated *KNOTTED1*-like cDNA represents the *STM* locus (Long et al. 1996). At the expression level, there is agreement between cells that accumulate *STM* mRNA and cells predicted to give rise to the shoot apical meristem based on histological analysis (Long et al. 1996). *STM* expression disappears in a domain of the meristem in the position of the incipient leaf

primordium, suggesting that its absence defines the position of the next leaf. A similar expression pattern had been observed before for *KNOTTED1* from maize (Jackson et al. 1994). Unfortunately, no loss-of-function phenotype (which gives the best clues to a gene's function) was available for the maize gene. Rather, *KNOTTED1* and some related genes had been identified by gain-of-function phenotypes. *KNOTTED1* is characterized by dominant mutations that affect the development of the leaf blade. Mutant leaves have localized zones of extra cell division in all cell layers, resulting in outgrowths or 'knots'. The *Knotted* gain-of-function phenotype is caused by ectopic expression of *KNOTTED1* in leaf veins of mutant plants (Smith et al. 1992). That ectopic expression of *KNOTTED1* in tobacco (*Nicotiana tabacum*) produces meristems on leaves had already suggested that this gene plays a role in meristem function, i.e. preventing differentiation or maintaining indeterminacy (Sinha et al. 1993). Similar gain-of-function phenotypes caused by unregulated cell division and expansion in the maize leaf result from ectopic expression of the *KNOTTED1*-like homeobox genes *ROUGH SHEATH1* and *LIGULELESS3* in the leaf (Schneeberger et al. 1995; Fowler et al. 1996). Thus, expression of these 'indeterminacy' genes in the leaf (which is a determinate plant organ) has to be downregulated for normal development to occur. In wild-type plants, expression of some of the maize homeobox genes predicts the site of leaf initiation (Jackson et al. 1994). While the absence of *KNOTTED1* expression marks the position of the incipient leaf (as already mentioned above), *ROUGH SHEATH1* and another homeobox gene, *KNOX3*, are expressed in a ring of cells just below that leaf. Thus, the precise temporal and spatial control of the expression of these genes may determine the spatial arrangement of leaves, i.e. phyllotaxy, as one of the major aspects of plant form.

Leaves arise as dorsoventral appendages from the flanks of the shoot apical meristem. They are initiated sequentially throughout the vegetative phase of shoot development. Leaves originate relatively uniformly as simple outgrowths, and subsequently exhibit divergent patterns of determinate growth. In their mature state, the leaves of most plants have a striking structural feature: they are considerably broader and longer than thick, so that relatively little tissue is needed to present a large area to incident light – clearly an adaptation to their role in photosynthesis. Leaf initiation occurs by coordinated changes in the polarity and rate of cell division and expansion within a group of founder cells on the flank of the shoot apical meristem.

Some plants produce alternative leaf forms, e.g. those exhibiting heteroblasty, a condition in which the juvenile phase of shoot development is distinct from the adult phase. For example, juvenile maize leaves are shorter and narrower than adult leaves, and they have epicuticular waxes not present on adult leaves, their epidermal cells are of different shapes to those of adult leaves and they lack the hairs present on adult

leaves. Leaves are subdivided into regions of differing identities, such as the sheath and blade regions of a grass leaf or the petiole and blade of a typical dicot leaf. The leaves of angiosperms come mainly in two basic arrangements: simple or compound. Simple leaves have a single blade, whereas compound leaves are composed of several independent blades (leaflets). Leaflets are distinguished from leaves, as only the latter form axillary buds. For a general review on the initiation and determination of leaves, see Smith and Hake (1992).

Many mutants are known where different aspects of leaf development are changed (for review, see Smith and Hake 1992).

For example, the pattern of heteroblasty is affected in the maize *Teopod* mutants, which prolong the expression of juvenile characteristics and structures, including juvenile leaves, throughout shoot development. The determination of regional identity within the leaf is changed in some other mutants. For example, pea plants homozygous for the *afila* mutation produce no lateral leaflets as in the wild-type, but instead develop a highly branched system of lateral tendrils. In contrast, *tendriless* mutants produce leaflet pairs instead of tendrils. *Tendrilled acacia* mutants produce a distal pair of subterminal tendrils but a terminal leaflet instead of a terminal tendril. Double mutants homozygous for both *afila* and *tendriless* show a novel phenotype consisting of a highly branched system of very small leaflets and no tendrils. In yet another type of mutant, leaf shape is strongly changed. In *phantastica* mutants of *Antirrhinum*, leaves produced at, and above, the fifth node are needle-like and show no evidence of dorsoventrality (Waites and Hudson 1995). They consist almost entirely of ventral tissue types (ventral epidermis, parenchyma, phloem and xylem), and lack the dorsal tissues present in wild-type leaves (dorsal epidermis, palisade and spongy mesophyll), suggesting that the *PHANTASTICA* gene product is necessary for the determination of dorsoventrality in snapdragon leaves (Waites and Hudson 1995).

Unfortunately, the cloning of any of the mentioned genes has not been reported up to now, and our knowledge about the molecular aspects of leaf development has remained quite poor. However, the first gene that regulates the transition from juvenile to adult shoot development in plants, *GLOSSY15* from maize, could be isolated recently and was shown to encode a putative transcription factor (Moose and Sisco 1996; see also Sect. 2.a.γ). In *glossy15* mutants juvenile leaf epidermal traits are replaced with adult ones beginning with the third leaf, but no other traits are affected. Thus, in contrast to the pleiotropic *Teopod* mutations, *GLOSSY15* acts specifically to promote a juvenile leaf epidermal cell identity.

Furthermore, some recent experiments, again involving the *KNOTTED1* gene of maize, have enabled interesting insights into the genetic conditions of simple vs compound leaves. Hareven et al. (1996) have reported that heterologous misexpression of *KNOTTED1* confers very different phenotypes on simple and compound leaves.

Ubiquitous transgenic misexpression of *KNOTTED1* in the compound wild-type tomato leaf confers dramatic additional orders of subdivision. Remarkably, such a ramification



is completely prevented in the tomato mutant *Lanceolate*, which has simple leaves. In line with this is the observation that when *KNOTTED1* was misexpressed in other dicotyledonous plants with simple leaves, such as tobacco and *Arabidopsis*, the leaves of these plants also remained simple, although severe morphogenetic alterations were caused (Sinha et al. 1993; Lincoln et al. 1994).

Based on their findings, Hareven et al. (1996) speculate that simple and compound leaves are determined by two different developmental programs and that the gene systems that condition them are conserved among species with simple and compound leaves.

### γ) Generative Development

When flowering plants have reached a critical age environmental signals may trigger a switch to floral development. The shoot apical meristem ceases production of leaf primordia and switches to the production of floral organs, i.e. sepals, petals, stamens and carpels. In most flowering plant species, the vegetative-to-floral transition takes place in two steps. First, a vegetative meristem is converted to or produces an inflorescence meristem. The inflorescence meristem then gives rise to determinate floral meristems which develop into flowers.

Inflorescence and flower development is probably the best understood morphogenetic process of higher plants. Accordingly, the genetics of flower development and related topics have recently been reviewed quite extensively (Ma 1994; Meyerowitz 1994; Okada and Shimura 1994; Weigel and Meyerowitz 1994; Haughn et al. 1995; Theißen and Saedler 1995). Because of space constraints, and trying to avoid redundancy, these articles are referred to for general reviews on the genes involved in inflorescence and flower development. In the following some of the most important recent advances are mentioned briefly, before exemplarily a schematic overview of the gene network that 'controls' *Arabidopsis* inflorescence and flower development is presented.

Among the 'highlights' of last year were reports on the cloning of *CYCLOIDEA* (Luo et al. 1996) and *CENTRORADIALIS* (Bradley et al. 1996), two genes from *Antirrhinum*. *CYCLOIDEA* is the first gene 'controlling' floral asymmetry that has been isolated. It is expressed in dorsal regions of developing floral meristems, where it affects growth rate and primordium initiation, and, at later stages, the asymmetry, size and cell types of petals and stamens. *CENTRORADIALIS* 'controls' the architecture of the *Antirrhinum* inflorescence. The wild-type *Antirrhinum* inflorescence is indeterminate, i.e. it does not form a terminal flower. In the *CENTRORADIALIS* mutant, however, after about ten axillary flowers have been initiated in the inflorescence a terminal flower is formed, so switching the inflorescence from an indeterminate into a determinate form. The molecular mode of action of the proteins encoded by both genes is unclear. *CYCLOIDEA* has no similarity to any characterized

gene in the database. *CENTRORADIALIS* is probably not a transcription factor, but is similar to animal proteins that associate with lipids and GTP-binding proteins. Another interesting gene that has recently been cloned is *BELL1* from *Arabidopsis*. *BELL1* turned out to encode a homeodomain protein that is distinct in sequence from members of the KNOTTED1 class (Reiser et al. 1995). The result of *BELL1* mutations is a loss of the identity of ovule integuments. Furthermore, the MADS-box genes *FBP7* and *FBP11* have been identified as 'master regulators' of ovule development in petunia (Angenent and Colombo 1996; and references therein). The floral homeotic gene *APETALA2* turned out to be the first member of a large family of genes encoding transcription factors, including *AINTEGUMENTA* (involved in ovule and flower development), *GLOSSY15* (see Sect. 2.a.β, Shoot Development) and *TINY* (Jofuku et al. 1994; Weigel 1995; Klucher et al. 1996; Moose and Sisco 1996; Wilson et al. 1996).

Also remarkable is the characterization of two *Arabidopsis* genes, *PERIANTHIA* and *SHORT INTEGUMENT1*, whose cloning has not yet been reported. In contrast to *Arabidopsis* wild-type flowers, that have four sepals, four petals, six stamens and two carpels, flowers of the meristic mutant *perianthia* show a pentamerous pattern of five sepals, five petals, five stamens and two carpels (Running and Meyerowitz 1996). From an evolutionary point of view it is interesting that the pentamerous *perianthia* pattern is characteristic for a number of plant families, but not the family Brassicaceae, which includes *Arabidopsis*. The *SHORT INTEGUMENT1* gene plays an important role in regulating both the development of ovules and the time of flowering (Ray et al. 1996), which led to the speculation that it also might have played a key role during the evolutionary origin of the flower (Roush 1996).

The many studies on the genetics of inflorescence and flower development 'flourishing' during the 1990s have culminated in the insight that these developmental processes are determined by a complex and quite conserved network of regulatory genes which 'control' the formation of the inflorescence meristem and its transition to the floral meristem, the initiation of floral organ primordia, and the floral organ identities (Fig. 2; for reviews, see Ma 1994; Okada and Shimura 1994; Theissen and Saedler 1995; Theissen et al. 1996). A genetic hierarchy has been proposed in which environmental signals trigger 'late flowering genes' that start reproductive development, perhaps by activating meristem identity genes. Meristem identity genes 'control' the transition from inflorescence to floral meristems. Cadastal genes analogous to the gap genes of flies set the boundaries of floral homeotic function. The homeotic organ identity genes specify the organ identity within the flower by activating 'realizators' which might be direct or indirect targets of the organ identity genes. In a current model, based on *Arabidopsis* genetics, three classes of homeotic gene activities ('homeotic functions') are proposed, called A, B and C. Within any one of the four flower whorls, expression of A alone specifies sepal formation. The combination AB specifies the

formation of petals and the combination BC specifies stamen formation. Expression of the C function alone determines the formation of carpels. The model also proposes that the A and C functions negatively regulate each other and that the B function is restricted to the second and third whorls independently of A and C functions. The genes providing the homeotic functions A, B, C could be cloned meanwhile from several plant species (a recent compilation was published by Theissen et al. 1996). Many other gene types are also involved in flower development, as reviewed elsewhere (Okada and Shimura 1994). Many of the genes regulate each other at the transcription level; thus, they constitute a gene

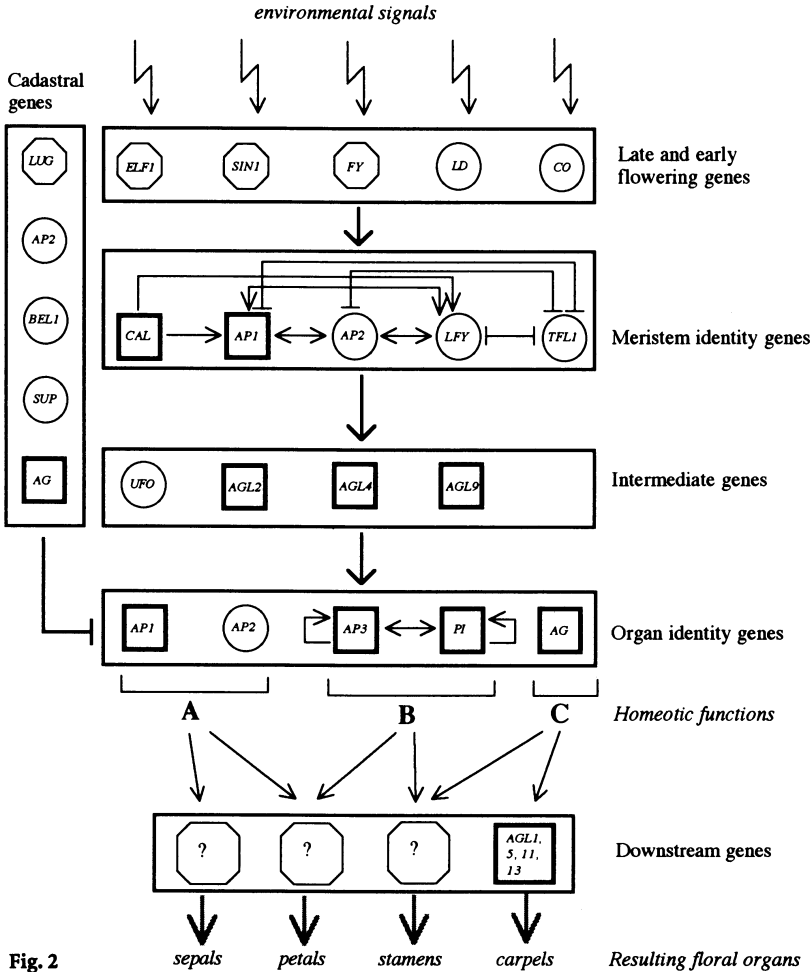


Fig. 2

network as defined by Wagner (1994). The authors have taken *Arabidopsis* as the best characterized model to summarize current knowledge about the gene network that 'controls' flower development. Figure 2 shows many of those genes which encode putative transcription factors of more than 60 genes that have been identified so far as being involved in *Arabidopsis* flower development (Okada and Shimura 1994). Our understanding of the interactions between these genes is symbolized by connecting lines and arrows. It is obvious that MADS-box genes play predominant roles in the gene network of flower development.

However, some other genes, encoding biochemically more diverse proteins, also play important roles – including *BELL1* (*BEL1*), encoding a homeodomain protein (Reiser et al. 1995), *APETALA2* (*AP2*), encoding the first representative of a large family of putative transcription factors (Jofuku et al. 1994, Weigel 1995), *SUPERMAN* (*SUP*) and *CONSTANS* (*CO*), encoding proteins containing one or two zinc-fingers, respectively (Putterill et al. 1995; Sakai et al. 1995), *LEAFY* (*LFY*), *UNUSUAL FLORAL ORGANS* (*UFO*) and *TERMINAL FLOWER* (*TFL*), which are the *Arabidopsis* orthologues of the *Antirrhinum* genes *FLORICAULA*, *FIMBRIATA* and *CENTRORADIALIS*, respectively (Weigel et al. 1992; Ingram et al. 1995; Bradley et al. 1996), and *LUMINIDEPENDENS* (*LD*), encoding a putative transcription factor with no similarity to members of the well-known families of transcription factors (Lee et al. 1994).

For a better overview, not all of the genes involved in flower development are shown in Fig. 2 (for reviews, see Ma 1994; Okada and Shimura 1994), and interactions (activation, repression) between the different hierarchy levels are depicted only globally (for some interactions between individual genes, see. e.g., Theißen and Saedler 1995). Many of the interactions shown are restricted to different tissues, e.g. *TFL1*, an inflorescence meristem identity gene, suppresses the activities of *LFY*, *AP1* and *AP2* in the inflorescence meristem, whereas *LFY*, *AP1* and *AP2*, as

Fig. 2. The genetic hierarchy that 'controls' flowering in *Arabidopsis thaliana*. Examples of different types of genes within each hierarchy level are boxed. MADS-box genes are shown as open squares with thick lines, non-MADS-box genes as circles, and genes whose sequence has not been reported up till now as octagons. That the MADS-box genes *AGL1*, *AGL5*, *AGL11* and *AGL13* are downstream of *AGAMOUS* (*AG*) is assumed because of their carpel-specific expression. The status of the other genes was taken from the literature, as cited by Theißen and Saedler (1995), Theißen et al. (1996) or within this review. Note that some genes have functions on different levels of the hierarchy, e.g. as cadastral genes, which set the boundaries of floral homeotic gene function, and as organ identity genes. Regulatory interactions between the different genes or blocks of genes are symbolized by arrows (activation), double arrows (synergistic interaction) or barred lines (inhibition, antagonistic interaction). Information about these interactions has been compiled from some recent review articles (for details, see Theißen and Saedler 1995; Theißen et al. 1996, and references therein). Abbreviations: ABC homeotic gene functions; *AGL1*, 2, 4, 5, 9, 11 and 13 *AGAMOUS*-LIKE GENE1, 2, 4, 5, 9, 11 and 13; *AP1* and 3 *APETALA1* and 3; *CAL* *CAULIFLOWER*; *ELF1* *EARLY FLOWERING1*; *LUG* *LEUNIG*; *PI* *PISTILLATA*; *SINI* *SHORT INTEGUMENT1*. The other abbreviations are explained in the text (Sect. 2.a.γ)

floral meristem identity genes, suppress *TFL1* activity in the floral meristem. In the case of the late and early-flowering genes, only a few of the many known genes are shown. Absence of lines or arrows between genes means that an interaction has not been experimentally demonstrated yet, not that it is not existing. In the case of the downstream genes, just one *octagon* or *square* is shown for every type of floral organ, though whole cascades of many direct target genes and further downstream genes probably exist in each organ. The carpel-specific genes shown (AGLs) are only putative examples. For the reasons mentioned above the figure is extremely simplified and preliminary.

#### b) Development of the Gametophyte

In contrast to lower plant species, in which the gametophyte is a free-living (e.g. ferns) or even the dominant generation (e.g. mosses), gametophytes of angiosperms are formed within specialized organs of the flower and are smaller and much less complex than the sporophyte. The male gametophyte (pollen) of flowering plants typically consists of only three cells and develops within the anther, whereas the typical female gametophyte (embryo sac) consists of seven cells and is a product of the ovule (for reviews on male and female gametophyte development of flowering plants, see McCormick 1993 and Reiser and Fischer 1993, respectively). A number of genes are known that are expressed during pollen or embryo sac development, but few mutants have been characterized in which gametophyte development is affected. The low number of female gametophyte mutations that have been described to date probably reflects the technical complexity of identifying the mutations rather than the actual number of genes involved (Reiser and Fischer 1993).

Little is known about genes that may actually 'control' gametophyte development. Recently, Chen and McCormick (1996) have described a male gametophytic mutation, termed *sidecar pollen*, in which some of the pollen produces an extra cell in the pollen grain. *Sidecar pollen* is possibly the first male gametophytic mutation to be described in *Arabidopsis*.

New avenues towards understanding gametophyte development of vascular plants may be opened by studying alternative model systems, such as the fern *Ceratopteris richardii* (Chasan 1992; Eberle et al. 1995; Hickok et al. 1995). In ferns, the gametophytic phase of the plant life cycle is easier to observe, manipulate and study than in flowering plants where it is surrounded by maternal sporophytic tissues. Therefore, the recent finding that some MADS-box genes are expressed in gametophytes of *Ceratopteris* (Münster et al. 1997) seems a promising starting point for future studies on gametophyte development of vascular plants.

### 3. Concluding Remarks

We have seen that two types of genes encoding transcription factors, the homeobox and the MADS-box genes, play pivotal roles in both animal and plant development (see Sect. 2; for MADS-box genes in animals, see Theißen et al. 1996, and references therein). That these genes have obtained predominant ontogenetic functions independently in the lineages of multicellular plants and animals suggests a certain 'suitability' of MADS-box and homeobox genes for specifying cell, tissue or organ identities during development. What could make their gene products so suitable for tasks in developmental 'control'? One aspect might be the capability of these proteins to interact with a diversity of other components, *cis*- as well as *trans*-acting factors (such as sequence elements in promoter regions and other transcription factors, respectively). That capability for molecular interactions facilitates the constitution of 'gene networks' i.e. sets of genes encoding transcription factors that mutually regulate each other's expression (Wagner 1994; Burstein 1995).

Gene networks with some similarity to neural and computational networks may be very suitable to 'control' pattern formation, at least theoretically. For example, a network model of developmental gene hierarchies has been developed that 'governs' the temporal and spatial hierarchy of pattern formation in *Drosophila*. The network units in that model are mostly evolutionary conserved transcription factors (Burstein 1995). Models of that type may be able to explain certain aspects not only of animal embryogenesis (Burstein 1995) but also of inflorescence and flower development (Theißen and Saedler 1995; Theißen et al. 1996).

However, during the course of this review many genes have been described which, upon mutation, affect pattern formation of plants, and by far not all of them encode putative transcription factors. Nevertheless, the authors called these genes collectively 'the molecular architects of plant body plans', and tentatively assumed that they 'control' development (thus, 'developmental control genes' would be an equivalent and more frequently used but less figurative term). Soon it became clear, however that in biochemical terms, these genes encode a highly diverse variety of products.

Among them are, besides transcription factors, a syntaxin-related protein that is involved in cytokinesis (KNOLLE), a nodulin-like protein involved in signalling (FOREVER YOUNG), a member of the Raf family of protein kinases (CONSTITUTIVE TRIPLE RESPONSE1), some genes whose sequence does not give immediate clues to their function (GNOM, CYCLOIDEA), etc. Even mutations in genes encoding ribosomal proteins may have specific effects on plant development (Van Lijsebettens et al. 1994; Chasan 1995).

How can genes that are so diverse, such as transcription factors, syntaxins, protein kinases or even ribosomal proteins, act together to constitute a 'program' that 'governs' the development of complex multicellular

organisms? A possible answer is that this is not what these genes actually do (genes encoding transcription factors included). One problem for geneticists is that the genes identified in genetic selections may not always directly be involved in the process of interest. Therefore, not all genes which, upon mutation, *affect* the formation of the body plan, may actually '*control*' its formation. However, the situation is probably even worse.

Although the concept that some genes '*control*' development, and that the genome encodes higher levels of organization, has pervaded contemporary thinking in molecular biology and related fields (the authors' review as well), it is not necessarily true in a strict (i.e. cybernetic or information-theoretical) sense. The study of animal development has provoked Nijhout (1990) to maintain that genes do not provide instructions for development, but that they just aid in supplying the material basis for development. According to Nijhout, development is a largely self-organizing process, a series of elaborate temporal and spatial interactions that are context-dependent. Genes are part of the network of interactions that occur during development rather than directors of the scenario.

The causal pathways of developmental events generally do not end at '*developmental control genes*', although their gene products might be required for these events to occur. The expression of these genes or the activity of their gene products must itself be controlled, either by other genes, by small inducing molecules such as organic compounds or ions, or by physical stimuli. Especially in the case of complex, non-linear systems with regulatory feedback loops – and that is what developing organisms are all about – the causal pathway might be endless (or undefined).

One example may illustrate that point. We have seen above (see Sect. 2.a.β, Shoot Development) that the positioning of leaf primordia (phyllotaxy) is correlated with (and thus may depend on) the downregulation of some homeobox genes. However, do these genes indeed '*control*' phyllotaxy? No, they are probably just instrumental in the determination of leaf primordia, because there must be some cause for the down-regulation to occur.

Two major hypotheses have been put forward to explain the positioning of leaf primordia (summarized by Jackson et al. 1994). The first suggests that inhibitory substances are responsible for positioning the new leaf primordium at a given spacing relative to preexisting leaf primordia. According to the second hypothesis, reorientation of cell wall cellulose microfibrils allows the change in growth polarity which is necessary for the outgrowth of the primordium. In both scenarios, it is a physicochemical process that determines leaf initiation. However, what then '*controls*' these events? Taken together, looking for a '*development control gene*' as the final cause might become a frustrating task.

According to Nijhout (1990), terms like '*developmental control genes*' and '*molecular architects of body plans*' are just evocative but inappropriate metaphors. The puzzling diversity of genes affecting pattern for-

mation in plants is clearly compatible with the view that these genes are part of the network of interactions during development rather than directors in the scenario. Their diversity thus simply reflects the diversity of interactions that occur during ontogeny.

If terms like 'molecular architects' and 'developmental control genes' are inappropriate metaphors, why then did such phrases become the jargon of developmental and evolutionary biology, not only in the animal, but also in the plant field? Possibly because the development of multicellular organisms is so complex, and so poorly understood, that communication about these issues is difficult. Therefore, we use some simple metaphors to facilitate the exchange of scientific ideas and results. Unfortunately, these metaphors imply some insight into causal relationships between genes and form, between genotype and phenotype, that we actually do not have, or that may be even wrong. That the authors (and, sorry, many others in the field) use such terms documents our ignorance concerning what development really is. Of course, we know already that development is a complex series of context-dependent temporal and spatial interactions, involving matter, energy and information, countless interior as well as environmental chemical and physical components. We also know that the network of gene activation and interaction seen during ontogeny is both the consequence of, and contributor to, development (Nijhout 1990). However, that is only the general picture, and we are still nearly completely ignorant about its extremely complex details. Since efficient techniques for cloning genes and analysing their expression and function are available, the genes involved in development can be studied relatively easily. Thus, we know relatively much about them and are tempted to assign them an overwhelming importance that they probably do not have. Developmental biology is not just a synonym for molecular genetics.

Although it might seem a surprising conclusion for a chapter entitled 'Molecular Architects of Plant Body Plans', in summary it turns out that plants (as well as animals) have no 'molecular architects of body plans' in a strict sense. However, they have many genes that substantially contribute to development. To study them will remain a challenging task for quite some time.

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## **Cell Biology and Physiology**

## Cytosymbiosis

By Thomas Friedl and Uwe G. Maier

Sitte and Eschbach (1992) mentioned in the last chapter on cytosymbiosis in *Progress in Botany* that "cytosymbiosis is defined as an intimate and long-lasting association of cells belonging to different taxa". Therefore, the living together of at least two heterospecific cells should be called a cytosymbiosis independent of the nature of the association. The interrelation can be somehow between intra- or extracellular parasitic forms and, at the other extreme, a non-parasitic endocytobiotic and intracellular association. Keeping this in mind, the definition for cytosymbiosis could be used for numerous examples of cell-to-cell interactions.

In this chapter the authors concentrate on cytosymbioses of general and botanical interest. The topics of the chapter are mostly theories or hypotheses concerning cytosymbioses found in lichens, algae as hosts or symbionts within other cells, and cytosymbioses leading to organelles/compartments in eucytes. The recent articles that are discussed represent only a short selection; other articles not cited here may be no less important.

### 1. Cytosymbiosis in Lichens

#### a) General Aspects of Lichen Symbiosis

Lichens are the symbiotic phenotype of nutritionally specialized fungi (mycobionts) that live as ecologically obligate biotrophs in symbiosis with algal and/or cyanobacterial photobionts (Honegger 1991a, Palmqvist 1995). The population of photobiont(s) is housed and maintained within the thallus of the mycobiont which, in most cases, is the quantitatively predominant exhabitant (Honegger 1996). Various definitions of the term "lichen" have been proposed and it is still a basic problem what actually constitutes a lichen (Hawksworth 1988, 1994b; Ahmadjian 1993a, Kappen 1994). The type of association between the bionts in a lichen is not uniform (Kappen 1993, 1994), but ranges from mutualism to mild forms of fungal parasitism on algal or cyanobacterial photobi-

onts (Ahmadjian 1993a,b, 1995; Honegger 1996). For a review of definitions of the term symbiosis, mutualism and parasitism see Lewis (1985), Smith (1993) and Sapp (1994).

Lichens may be regarded as "a prime example of one of the most successful symbioses in nature" (Nash 1995). The oldest unequivocal fossil lichen has been reported from thin sections of Early Devonian (400 million years old) Rhynie chert (Taylor et al. 1995). The photobiont appears to be a cyanobacterium which shares numerous morphological features with several extant unicellular cyanobacteria, e.g. *Gloeocapsa* or *Chroococcidiopsis*. About 8% of all terrestrial ecosystems are lichen dominated (Larson 1987) and in some extreme environments lichens constitute the only photoautotrophic form of life (e.g. Friedmann 1993). The ecological success of lichens may be mostly due to the fact that they are poikilohydrous systems (review: Green and Lange 1994) which can withstand dramatic wetting and drying cycles. Lichens exhibit a wide variety of fascinating ecological and ecophysiological features that have been described in a vast body of recent literature, which to review, however, is beyond the scope of this chapter. Lichens can also be reliably used in monitoring air pollution (reviews: Gries 1996; Henderson 1996).

Lichens exhibit an intriguing carbon metabolism which operates within and between two closely associated symbiotic partners with often contrasting metabolic features. The various fascinating aspects of carbon fixation, secondary compound chemistry and nutrient exchange between the bionts have been discussed in many recent reviews (Feige and Jensen 1992; Honegger et al. 1993; Fahselt 1994a,b; Palmqvist et al. 1994a,b; Palmqvist 1995).

Various other unique characteristics of the lichen symbiosis, e.g. morphology and morphogenesis, reproductive strategies and taxonomy, can also not be covered by this short chapter. The interested reader is referred to the various excellent recent reviews of Honegger (1991a,b, 1992, 1993, 1996), Hill (1994) and the text books by Galun (1988), Ahmadjian (1993a) and Nash (1996).

## b) Mycobionts

Lichen-forming fungi are a polyphyletic group of nutritionally specialized fungi and there are no fundamental differences between lichenized and non-lichenized fungi (Hawksworth 1988; Honegger 1991a,b; Ahmadjian 1995). This long held view has recently been confirmed and new arguments have been added to it by small subunit (SSU) rDNA sequence comparisons (Gargas et al. 1995b; Jørgensen 1995). One in five of all known fungi are lichen formers composed mainly of ascomycetes (about 46% or 16 orders of Ascomycotina; Hawksworth 1988; Honegger 1996), a few basidiomycetes (about 0.3% or 2 orders of Basidiomycotina) and



some conidial fungi (about 0.3% or 2 orders of Deuteromycotina; reviews: Hawksworth and Honegger 1994; Honegger 1996). A large number of lichen mycobionts can be cultured in the aposymbiotic state (Ahmadjian 1993a; Stocker-Wörgötter 1995). The symbiotic phenotype of lichen mycobionts may reach structures of a considerable morphological and anatomical complexity (Honegger 1993; Büdel and Scheidegger 1996). However, a mycobiont cannot produce a lichen thallus alone. It is the photobiont that provides the stimulus that causes fungal transformation into a thallus, but what the signals are from the photobiont is not known yet. Possible kinds of signals that occur in a lichen symbiosis have been discussed by Ahmadjian (1992, 1993a).

Most mycobionts associate with a rather narrow range of compatible algal or cyanobacterial photobionts, i.e. several species of a photobiont genus or, more rarely, even with several genera of photobionts (Ahmadjian 1993b; Friedl and Büdel 1996). On the other hand, one species of photobiont can occur in widely different lichens. Galun and Kardish (1995) discuss the possible role of lectins in the discrimination between compatible and incompatible photobionts at early stages of the lichen formation. About 3–4% of lichen forming fungi associate with a second, cyanobacterial photobiont to exploit their nitrogen-fixing capacity. These triple symbioses may form two structures of strikingly different appearance attached together or are found as independent lichen thalli; their forms are determined by the nature of the photobionts (photomorphs; Laundon 1995). DNA analysis confirmed that the two seemingly very disparate photomorphs share the same mycobiont (Armaleo and Clerc 1991). Green algal lichens may also form facultative or obligate associative symbioses with aposymbiotic nitrogen-fixing cyanobacteria ("cyanotrophic lichens"; Poelt and Mayrhofer 1988).

Taxonomy of lichens today mainly relies on generative and vegetative morphology of the mycobiont and secondary compound chemistry (mostly absent in lichens associated with cyanobacteria). Many comprehensive reviews have been published recently (e.g. Rambold and Triebel 1992; Jahns 1993; Sipman 1993, 1995; Hawksworth 1994a; Theler 1996). Only recently, molecular analyses, i.e. sequence comparison of SSU rDNAs and their introns, have been performed to infer evolutionary relationships among lichenized fungi (e.g. DePriest and Been 1992; Gargas and Taylor 1992; DePriest 1993a,b, 1994, 1995; Gargas et al. 1995a, Grube et al. 1995, 1996; Lutzoni and Vilgalys 1995; Beard and DePriest 1996).

### c) Photobionts

Overviews of genera of eukaryotic algae and cyanobacteria that are known to occur in lichen symbiosis have been presented by several

authors (Tschermak-Woess 1988; Büdel 1992; Gärtner 1992; Ahmadjian 1993b; Ettl and Gärtner 1995; Friedl and Büdel 1996). In terms of their occurrence, the most important photobionts are the green algae *Trebouxia* (unicellular) and *Trentepohlia* (filamentous) and the filamentous cyanobacterium *Nostoc*. Recent SSU rDNA analyses showed lichen symbiosis being of multiple origins within the green algae (Chlorophyta). *Trebouxia* as well as other coccoid lichen photobionts are evolutionarily closely related to non-lichenized terrestrial green algae; they form independent lineages within a monophyletic group of the Chlorophyta, the class Trebouxiophyceae (Friedl 1995). For *Trentepohlia*, preliminary rRNA analyses suggest its phylogenetic position within the class Ulvophyceae (Zechman et al. 1990; Chapman and Buchheim 1991) which is independent of the Trebouxiophyceae (Friedl 1995, 1996). Phylogeny of the cyanobacteria as inferred from SSU rRNA sequence analyses has been reviewed by Wilmotte (1994) and Nelissen et al. (1995, 1996). The phylogenetic position of most cyanobacterial photobionts and their relatedness with non-symbiotic taxa is not known yet.

The most common aerophilic algae (review: Ettl and Gärtner 1995) are not compatible photobionts. The question whether *Trebouxia* spp., the most frequently found lichen photobionts, occur aposymbiotically in nature has remained a lively discussed debate among lichenologists. Using immunological methods, Mukhtar et al. (1994) identified *Trebouxia* cells with the same cell surface antigenic properties as the *Trebouxia* photobiont of the lichen *Xanthoria parietina* among algae cultivated from rock surfaces exposed to fire and that were free of lichens. Gärtner (1994) reported *Trebouxia* among common green algae on tree barks, and two of all described *Trebouxia* spp. may have been found outside of lichens (Ettl and Gärtner 1995).

The rare reports on *Trebouxia* found outside of lichen thalli (summarized in Mukhtar et al. 1994) may indicate that *Trebouxia* is at least not a common member of the free-living algal community. Other lichen photobionts, e.g. *Dictyochloropsis* (e.g. Nakano and Isagi 1998; Tschermak-Woess 1995), *Trentepohlia* (e.g. Nakano 1988; Nakano and Ihda 1996) and the cyanobacteria *Nostoc* and *Chroococcidiopsis* (Büdel 1992), are frequently found in the aposymbiotic state (Friedl and Büdel 1996). However, it has not been established yet whether symbiotic and aposymbiotic members of photobiont genera are genetically distinct from each other, e.g. at the species level. These intriguing questions should be addressed by molecular studies, especially in the cyanobacteria, where a clear species concept is still lacking (for discussion see Büdel 1992, Wilmotte 1994).

## 2. Cytosymbiosis Concerning Algae

### a) Various Algae as Partners in Symbiotic Associations

Still one of the best compilations of symbiosis concerning algae as partners of other cells has been edited by Reisser (1992a). It was demonstrated that algae are apparently very successful in symbiotic systems formed by eukaryotic cells. Besides the *Chlorella/Paramecium* system (e.g. Reisser 1992b; Reisser and Widowski 1992) and the lichen associations (this chapter), which are most likely the best known eukaryotic systems concerning intracellular or inhabitant algae, many other associations are described. In freshwater habitats these include *Chlorella* or other Chlorophyta which are found in Protozoa, Porifera, Coelenterata, Rotatoria, Mollusca and Turbellaria. In marine habitats there are plenty of symbioses between algae and, as their hosts, Foraminifera, Radiolaria, sponges, Coelenterata, ascidians and other (e.g. Reisser 1992c; Reisser and Widowski 1992). Symbiotic eukaryotic algae can be rhodophytes, chlorophytes, dinoflagellates, chrysophytes, cryptophytes, prymnesiophytes and diatoms (Anderson 1992; Lee 1992a,b; Wilkinson 1992).

### b) Symbiotic Associations Concerning Dinoflagellates

In dinoflagellates, many symbiotic associations are described. Dinoflagellates occur as hosts as well as symbionts. Traditionally, dinoflagellates as symbionts are called "Zooxanthellae" when found in coelenterates (e.g. Battey 1992). Dinoflagellates as hosts cells can harbour different types of ecto- or endocytic symbionts; the symbiosis is sometimes fully synchronized and the intracellular partner dependent on the host. Thus, dinoflagellates are the playground of nature for "experimenting" with various stages of symbiosis between two cells (Schnepf 1992). The partners of dinoflagellates may also be of prokaryotic nature, endocytic or ectocytic bacteria or "blue-green algae" (cyanobacteria). On the other side, several dinoflagellates are described as the hosts for chromophytes, cryptophytes or chlorophytes (e.g. Larsen 1992; Schnepf 1992; as reviews: Elbrächter and Schnepf 1996). Again, the association may be endocytic or ectocytic in these cases. Furthermore, even endoparasitic forms are described in which dinoflagellates are parasites within other dinoflagellate cells (e.g. Fritz and Nass 1992).

A striking and unique strategy of the dinoflagellates to gather a photosynthetic organelle are the so-called kleptoplastids. These are plastids that have been "stolen", i.e. they are the result of an endocytotic uptake of a foreign plastid-containing cytoplasm. In this process, plastids from the "food cytoplasm" are retained and temporarily used as photosynthetic organelles (Schnepf et al. 1989; Schnepf 1992, 1993).

Only in a few cases the physiology, molecular evolution and state of dependence of the partners on one another have been studied in dinoflagellate associations as well as in all other kinds of symbioses concerning algae. It will be a fascinating field for biochemists, molecular and cell biologists to study the different aspects of cytosymbiosis, thereby learning about the strategies that have been and will be used for symbiosis.

### **3. Evolution of Eukaryotes Involves Different Endocytobiotic Events: Cytosymbiosis and the Chimaeric Nature of Eucytes**

Our knowledge about the evolution of eukaryotic cells (eucytes) has increased rapidly in the last 5 years. Modern biochemistry, cell and molecular biology have accumulated an enormous amount of data demonstrating a possible (nucleus, peroxisome, basal body), most likely (hydrogenosomes) and definitive (plastids, mitochondria) endocytobiotic origin of some major compartments/organelles of eucytes.

#### **a) Cell Nucleus**

The cell nucleus is surrounded by a double membrane, the nuclear envelope. The establishing of such a compartment, harbouring the genome of the cell, was discussed in a review by Lake and Rivera (1994). In the karyogenic hypothesis (Lake and Rivera 1994) the nucleus is acquired by an unspecific intracellular segregation process, which led to a nucleus without any endocytobiotic events. However, this postulates a mechanism which is hard to reconstruct with the knowledge of modern cell biology. Therefore, it is thought alternatively that the double membrane of the nucleus may have arisen from endocytobiosis. This is called the endokaryotic hypothesis, originally postulated at the beginning of this century (e.g. Mereschkowsky 1910). Modern molecular phylogeny and cell biology have concentrated on the question of endocytobiosis concerning the cell nucleus, but the answers were non-uniform. Sogin (1991) has reinitiated the discussion about the possible endosymbiotic origin of eukaryotic nuclei. In his still discussed paper he postulated the origin of the eukaryotic nucleus in an archaebacterial line, thereby explaining the phylogenetic proximity of some eukaryotic proteins with their archaebacterial homologues. In his view, the progenitor of eukaryote cells, the proto-eukaryote, engulfed and stably integrated an archaebacterial cell, creating the first chimaeric cell. The new nucleus was created by the transfer of genes that are typical for eukaryotes, e.g. genes for cytoskeleton proteins, into the former archaebacterial cell. This hypothesis could explain the gene content of extant eukaryotes and at least some phylogenetic proximities of archaebacterial and eukaryotic

protein genes. On the other hand, however, data have accumulated which allow space for the interpretation that the cell nucleus may not only be of archaeobacterial and eukaryotic sources. For example, phylogenetic analyses of the heat shock protein Hsp70 by Gupta and Singh (1994) have resulted in a different explanation concerning evolution of early eucytes. Although they deduce from their phylogenetic trees that the origin of the eukaryotic nucleus was an endocytobiotic event, they found evidence that the chimaeric eukaryote may also have been built up by an intracellular cytosymbiosis between a Gram-negative eubacterium and an archaeobacteria (Gupta et al. 1994; Gupta and Singh 1994).

However, a close inspection of a great number of available protein data by Doolittle and Brown (1994) indicates that depending on the molecular marker different phylogenetic affiliations can be seen. Moreover, having in mind that genes can also be integrated in a nucleus by invading nucleic acids (horizontal gene transfer), the evolution of kingdoms from a single molecular marker alone cannot be judged. Therefore, the picture of the evolution of early eucytes is, at this point, still confusing. It is anticipated that new data, especially those from genome projects (e.g. Johnston 1996), will resolve these questions in the near future.

#### b) Basal Bodies and Peroxisomes

For the possible endocytobiotic origin of basal bodies (e.g. Margulis 1981) a definitive proof is still missing. Contrary facts are still the detection of the *uni* linkage group which is genetically involved in flagellar movement and the impossibility to stain DNA in the ultrastructure of this region (Hall et al. 19889; Johnson and Rosenbaum 1990; Johnson and Dutcher 1991). Also, when the detection of DNA in the region of basal bodies may continue to fail, a cytosymbiotic origin appears still to be possible. To solve the problem of the evolution of basal bodies remains an intriguing task for molecular biology of the next years.

In the case of peroxisomes several arguments suggest an endocytobiotic origin (e.g. the formation of new peroxisomes by division of existing ones, or budding from a peroxisomal reticulum; see Borst 1989; Cavalier-Smith 1993a). The isolation of genes of proteins located in peroxisomes and the inference of their phylogenetic history will certainly reveal unambiguous conclusions. First results support the hypothesis of the symbiotic origin of peroxisomes (e.g. Igual et al. 1992). Furthermore, mechanisms for the targeting of peroxisomal proteins to and into the compartment are becoming cleared and their better understanding will also help to resolve the phylogenetic puzzle. Interestingly, there exists a connection between mitochondria and peroxisomes. Nuclear-located single-copy protein coding genes have been detected with their gene products being located in the peroxisomes and in the mitochondria

as well (Elgersma et al. 1996; Ashmarina et al. 1996; Oatey et al. 1996). However, a single gene that operates for the transport into peroxisomes and mitochondria cannot be a definitive proof of the phylogenetic relationship of these two compartments.

### c) Mitochondria, Hydrogenosomes and Plastids

The most intimate forms of cytosymbiosis in eukaryote cells that may be regarded as definitely proven are found in mitochondria, plastids and, as it has been shown only recently, in hydrogenosomes. In nearly every extant eucyte, regardless of the presence or absence of mitochondria and heterotrophy or photoautotrophy, at least one endosymbiotic event led to intracellular organelles that are double membrane-bound (exception Archaezoa, Cavalier-Smith, 1993b). While in mitochondria and plastids remnants of the symbiotic genome are still present, in hydrogenosomes all symbiotic DNA is lost or transferred into the nucleus, the host genome.

In terms of phylogeny, evidences for either a monophyletic or a polyphyletic origin are very important. To avoid confusion, these terms are used in this chapter in the sense that monophyly is the origin of present taxa by one single endosymbiosis, i.e. one host cell has engulfed one symbiont, and all extant taxa are of one single origin. Polyphyly is used when (1) one host cell has engulfed several taxonomically different cells and established them as symbionts; (2) different hosts harbour several taxonomically identical symbionts; or (3) different hosts have engulfed several different symbionts.

### $\alpha$ ) Mitochondria

A large data set exists demonstrating the origin of mitochondria from alpha purple bacteria (e.g. Gray 1992, 1993; Brennicke et al. 1996). Recent data strongly suggest the monophyletic origin of mitochondria (e.g. Gray 1993; Burger et al. 1996 and references herein). Research on mitochondria in terms of phylogenetic studies is driven by projects dealing with the sequence of whole mitochondrial genomes. Due to their mostly small size an increasing number of mitochondrial genomes is sequenced or still underway (e.g. for phototrophic cells or protists: Ode et al. 1992; Wolff et al. 1994; Burger et al. 1995; Leblanc et al. 1995; Unseld et al. 1997; for other mitochondrial whole genome sequencing projects see the Organelle Genome Megasequencing Programme at their URL <http://megasun.bch.umontreal.ca/>). Beside advantages that are particularly helpful for clarifying mitochondrial evolution, comparing of whole genomes has led to a broad range of interesting results. For example,

differential loss of protein encoding genes from the mitochondrion genome is described for several taxa (review: Brennicke et al. 1996). More recently, Burger et al. (1996) have shown that the transfer of genetic material from *sdh*-genes from the mitochondrial to the host genome may have occurred several times. Furthermore, an inspection of mitochondrial 5S rRNA has shown another aspect of dynamic gene loss during the diversification of eukaryotic lineages (Lang et al. 1996).

Gene transfer is not only observed from the mitochondrial genome into the nucleus. Invasion of DNA into the mitochondrial genome has been described, including DNA from the plastid genome (e.g. Stern and Lonsdale 1982; Schuster and Brennicke 1988) as well as retrotransposons and rRNA-genes of nuclear origin (review: Brennicke et al. 1996). However, not only gene transfer into the mitochondrion genome may occur. In the case of some tRNA genes, which have invaded the mitochondrion via the plastid, foreign gene products are now utilized in the mitochondrion (Joyce and Gray 1989).

Introns of the group I and group II types are common in plant mitochondria (e.g. Brennicke et al. 1996), the latter type being restricted to organelles in eucytes and to eubacteria related to the prokaryotic ancestors of mitochondria and plastids (Knoop and Brennicke 1994; Knoop et al. 1994). Recently, it was shown that trans-splicing, the connection of exons from independent RNA molecules, evolved most likely from cis-splicing group II introns and the establishment of land plants was predated. (Malek et al. 1997).

RNA editing is the alteration of primary transcripts in mRNA as well as in tRNAs. This process is very common in plant mitochondria. Malek et al. (1996) demonstrated that RNA editing may be older than previously thought and has not been developed late in green plant evolution since it has been found in liverworts, true mosses and hornworts as well. RNA editing activity appears to have been lost and gained more than once in green plant evolution (see Brennicke et al. 1996).

## β) Hydrogenosomes

Hydrogenosomes are double membrane-bound compartments. In these organelles, pyruvate is fermented, and broken down to acetate, CO<sub>2</sub> and hydrogen (Müller 1988). It has been argued that hydrogenosomes are the pendant of mitochondria in some ancient eukaryotes (e.g. Cavalier-Smith 1993a). Hydrogenosome-like organelles are also found in some ciliates and fungi (e.g. Finlay and Fenchel 1989; Müller 1993). Whenever an organism contains a hydrogenosome, no mitochondria and peroxisomes can be found. However, presence of mitochondria is normally connected to that of peroxisomes. An independent endocytobiotic event for the hydrogenosomes has been discussed, and that mitochondria and

hydrogenosomes were descendants of one intracellular symbiosis (e.g. Cavalier-Smith 1987; Müller 1993; Palmer 1997). In phylogenetic investigations of trichomonads, hydrogenosomal Hsp70, Hsp60 and Hsp10 DNA sequences have been found to suggest a common phylogenetic origin of hydrogenosomes, kinetoplastids and mitochondria (Bui et al. 1996; Germot et al. 1996; Roger et al. 1996). Therefore, it is likely that the conversion of mitochondria to hydrogenosomes is an adaptation to anaerobic conditions and not a divergency of some ancient organisms before the radiation of eukaryotes after establishing mitochondria.

Hydrogenosomes do not contain DNA which is in contrast to mitochondria. However, an inspection of typical mitochondrial genes, like Cox subunits, shows that they code for biochemical functions which are not essential in hydrogenosomes (e.g. Cavalier-Smith 1987). Therefore, all genes of the symbiont whose coding capacity has to be maintained are located in the nucleus. Hydrogenosomes are the first organelles without any genome, from which the symbiotic origin seems to be proven.

#### γ) Plastids

Research concerning the evolution of phototrophic eukaryotes has been very successful in recent years. The progress has been reviewed several times, e.g. Martin et al. (1992), Palmer (1993), Douglas (1994), Bhattacharya and Medlin (1995) and Melkonian et al. (1995). Following the view of the endosymbiont theory and that eukaryotes are chimaeric cells (Mereschkowsky 1910; Margulis 1981), phylogeny should be investigated for the host cell and the symbiont independently. Besides general aspects of evolution, conclusions supporting a mono- or a polyphyletic origin of phototrophic eucytes are very interesting. However, the majority of available data cannot support a polyphyletic origin of the plastids.

Today, phylogenetic studies of plastid evolution are mostly done by techniques of molecular biology. For inferring relationships among kingdoms, subkingdoms and phyla (Cavalier-Smith 1993b), routinely the SSU and LSU (large subunit) rRNA genes are amplified, sequenced and phylogenetically analysed using various statistical approaches. The phylogenetic trees fail to fully resolve the very early divergency of eukaryotic lineages due to the low statistical reliability. However, major evolutionary trends as well as the later grouping of the taxa can still be demonstrated by these methods (e.g. Cavalier-Smith 1993b; Van de Peer et al. 1996a).

The host cells of photosynthetic groups diverge in the chlorophytes and embryophytes, euglenophytes, chlorarachniophytes, rhodophytes, haptophytes, cryptophytes, glaucophytes, heterokont algae (Stramenopiles: Leipe et al. 1994) and the Alveolata. In contrast to the host cells,



plastid evolution is reflected by "green" plastids (green algae, embryophytes, euglenophytes, chlorarachniophytes), "red" plastids (rhodophytes, heterokont algae, cryptophytes, haptophytes) and the cyanelles found in glaucophytes and most likely in *Paulinella chromatophora* (Bhattacharya et al. 1995a). However, phototrophic dinophytes acquired their plastids by different mechanisms (review Schnepf 1993). In order to understand the evolution of all these taxa as well as the relationships within these groups, a clear distinction between host and plastid evolution is important, as well as the knowledge of how the host cells obtained their capability of phototrophic growth, i.e. through primary or secondary endocytobiosis.

The traditional scheme of evolution of mitochondria and plastids, explained by the endosymbiont theory, includes an engulfment and stable integration of a prokaryotic cell as an organelle in the host cell (Margulis 1981). In the case of phototrophic eukaryotic organisms, a further evolutionary strategy led to the ability of oxygene photosynthesis, called the secondary endocytobiosis (e.g. Gibbs 1981; McFadden and Gilson 1995; Palmer and Delwiche 1996). In this scenario, a phagotrophic eukaryotic cell has engulfed another cell of eukaryotic and phototrophic nature. In the course of intracellular coevolution, the eukaryotic symbiont parts were reduced or eliminated. Today it is established that those algal groups whose plastids are surrounded by four membranes (so-called complex plastids: Sitte 1993) evolved through secondary endocytobiosis. Still accumulating data demonstrate that plastids with three boundary membranes are the remnants of a eukaryotic cell withing another cell (e.g. Van de Peer et al. 1996a; Köhler et al. 1997).

Different steps of intracellular evolution can be distinguished by the morphology of the plastid. In chlorarachniophytes and cryptophytes, remnants of the symbiont's eukaryotic components are maintained, the cytoplasmic membrane, 80S ribosomes that are embedded in cytoplasm, the periplastidal compartment, and a reduced cell nucleus, the nucleomorph (McFadden and Gilson 1995). A further step in intracellular coevolution of two eucytes is seen in heterokont algae and haptophytes. Here, there are no eukaryotic cell components of the eukaryotic symbiont maintained except for the remnants of the former cytoplasmic membrane. Therefore, the plastid is bounded by four membranes (Melkonian et al. 1995).

Two algal groups, the Euglenophyta and most of the phototrophic Dinophyta (photosynthetic members of the Alveolata: Leipe and Hausmann 1993; Van de Peer et al. 1996b), contain plastids that are surrounded by three membranes. This could be explained by the elimination of one of the four boundary membranes mentioned above. If this proves holds true, Euglenophyta and Dinophyta evolved in secondary endocytobiosis, too. Another possibility is that plastids bounded by three membranes represent ancient types of organelle evolution.

As mentioned above, the early divergencies of the Eukaryota (Cavalier-Smith 1993b) in different lineages cannot be resolved by the standard statistical techniques of molecular evolution (e.g. Cavalier-Smith 1993b; Van de Peer 1996a,b). However, soon after the progenitors of recent taxa have arisen in the phylogenetic trees the molecular analyses show a consistent picture for the further progressing evolution. The host cells of green algae, red algae, heterokont algae and the Alveolata form independent lines and are per se monophyletic. This may also hold true for the host cell of the Chlorarachniophyta (Cavalier-Smith et al. 1996; Van de Peer et al. 1996a). Recent findings of Bhattacharya and the group of M. Melkonian further demonstrate that the chlorarachniophyte host lineage contains different other phototrophic and heterotrophic members (Bhattacharya et al. 1995a; furthermore Cavalier-Smith 1995). In the Cryptophyta, the host cell may be somehow closer related to the glaucophytes. However, significant statistical support for this grouping is still lacking in most phylogenetic trees (Bhattacharya et al. 1995b; Van de Peer et al. 1996a).

Eukaryotic symbiont partners in algae that have evolved in secondary endocytobiosis can be distinguished into the cryptophytes and chlorarachniophytes, as seen from the presence of a nucleomorph genome which contains rDNA-operons in the two groups. Earlier analyses have shown a confusing picture of nucleomorph evolution where cryptophyte and chlorarachniophyte nucleomorphs are grouped in one cluster (e.g. Cavalier-Smith et al. 1994). However, this has been shown to be a statistical artifact due to the high evolutionary rates in these lineages (Felsenstein 1978; Olsen 1987; Van de Peer et al. 1993). The problem has been resolved by new tree building methods (Van de Peer et al. 1996; see also Cavalier-Smith et al. 1996). A relationship of the eukaryotic symbiotic partner in cryptophytes with the red algae has been established now, while the symbiont of the chlorarachniophytes is related within the green algae, possibly to some *Chlorella* spp. (Van de Peer et al. 1996a).

The considerable progress concerning the evolution of plastids is mostly due to the sequencing of whole plastid genomes which demonstrate the actual gene content of these genomes (Ohya et al. 1986; Shinozaki et al. 1986; Wolfe et al. 1992; Hallick et al. 1993; Wakasugi et al. 1994; R. M. Maier et al. 1995; Reith and Munholland 1995; Kowallik et al. 1995; Stirewalt et al. 1995). The studies are certainly of outstanding impact for inferring evolutionary relationships especially when compared with a recently determined cyanobacterial genome (Kaneko et al. 1996). At this point, only some of the most intriguing features can be mentioned: the plastids of the cyanelle of glaucophytes and the plastomes of red and heterokont algae seem to be different in gene content compared with the plastomes of *Euglena* and the embryophytes. The plastome of the red alga *Porphyra purpurea* contains many more genes than that of green plastids, and cyanelles are somehow more primitive.

Interestingly, there may exist some mechanism that appears to be restricted to those plastids containing chlorophyll b. This may also be true for RNA editing (e.g. Hoch et al. 1991) as well as for the occurrence of introns in chlorophyll b containing plastids. Euglenophytes, with their fascinating twintrons (Copertino and Hallick 1993; Copertino et al. 1994), green algae and embryophytes harbour a set of introns, whereas in non-chlorophyll b algae plastomes intervening sequences seem to be lacking (however, note the two exceptions: Bernard et al. 1992; U. G. Maier et al. 1995).

Genes which are missing in the plastomes and whose gene products are important for the maintenance of the plastid should be located in the nucleus. They are transferred by gene transfer mechanisms into the nucleus. However, in some cases the genes were lost and functionally replaced by a nuclear homologue or a retoured mitochondrial, nuclear located gene (e.g. Martin et al. 1990, 1996; Keeling and Doolittle 1997).

In future, whole sequencing of plastomes will have a still greater impact on evolutionary studies. Also, the increased computer facilities will aid in order to give a convincing answer for the mono- or polyphyly question. At present studies of a single gene have tried to explain the evolution of plastids. Phylogenetic trees for the plastids of higher plants are mostly done by using LSU RuBisCo as the marker molecule (Chase et al. 1993). However, RbcL phylogenies show a discrepancy to SSU rRNA-based studies when used for reconstruction of the algal plastid evolution (Martin et al. 1992). This could be caused by an ancient gene transfer of the RuBisCo operon in the ancestor of red algae. A more convincing picture is provided, for example, by phylogenies using TufA (e.g. Delwiche et al. 1995; Köhler et al. 1997) and SSU rRNA (e.g. Helmchen et al. 1995; Van de Peer et al. 1996a). Again, with new methods it is possible to demonstrate that two monophyletic lines exist, a chlorophyll a+b and a chlorophyll a+c/chlorophyll a+phycobilins line (Van de Peer et al. 1996a). Therefore, phylogenetic investigations with plastid SSU rDNA sequences in comparison with host SSU rDNA-trees show the importance of investigating symbiont and host evolution independently.

As mentioned above, the Alveolata contain one phototrophic group, the dinoflagellates. Unfortunately no data about sequences from the plastid are available for this group. These fascinating algae may certainly have a somehow different evolution regarding their plastids. Only recently, dinoflagellate plastids were found to harbour a form II RuBisCo that is normally not used in cyanobacteria (Morse et al. 1995; Whitney et al. 1995; Rowan et al. 1996). Furthermore, the corresponding gene for RuBisCo is nuclear and not located in the plastid.

#### 4. Plastids in Human Parasites

The Apicomplexa, a phylum of the Alveolata, comprises unicellular endoparasites that are of considerable medical significance as they are the causative agents for a wide variety of human diseases including malaria. These organisms possess a specialized apical structure for penetration of the host cell, the so-called apical complex. Cell biological investigations have demonstrated a compartment which is called a "Hohlzylinder" (Scholtyssek and Piekarski 1965), spherical bodies (Aikawa 1966), vacuoles (Rudzińska and Vickerman 1968) and double-walled vesicles (Hackstein et al. 1995). The "Hohlzylinder" is surrounded by two or more boundary membranes (McFadden et al. 1996). Genetic analysis of *Plasmodium* cells indicates two different extrachromosomal nucleic acids, an A/T-rich linear molecule of tandemly arranged 6-kb elements and a circular molecule of 35 kb (Wilson et al. 1991). The 6-kb element represents the mitochondrial genome (Wilson et al. 1992), whereas the 35-kb chromosome is similar to plastid genomes regarding its gene content and arrangement (Wilson et al. 1996; Köhler et al. 1997). For example, the 35-kb chromosome encodes for rRNAs, which are arranged as inverted repeats and harbours an rpo-operon, in which rpoC is encoded as two independent subunits (Wilson et al. 1996).

The puzzle has been elegantly resolved by in situ localization of the 35-kb element (McFadden et al. 1996; Köhler et al. 1997). Independent studies revealed that in different Apicomplexa the "Hohlzylinder" is the cellular localization of the circular 35-kb chromosome. Therefore, the "Hohlzylinder" is a remnant of a plastid, harbouring a semiautonomous plastid-like genome. Recent phylogenetic analyses concerning the TufA sequence of different Apicomplexa plastid genomes indicate a branching together with *Euglena* and the green algae (Köhler et al. 1997). Therefore, these studies strongly suggest that the plastid of Apicomplexa has evolved by secondary endocytobiosis in which a green alga was established into the progenitor of the apicomplexan host cell. In the course of intracellular evolution the ability of photosynthesis was lost, most probably caused by the parasitic life style of the cells.

#### 5. Cytosymbiosis and the Apicomplexa Doctrine

What can we learn from the findings in Apicomplexa? As indicated by Sitte and Eschbach (1992), cytosymbiosis can lead to different more or less stable associations of cells. However, if an association is stable and has non-parasitic features, at least one of the partners is dependent on the other. In the case of intracellular cytosymbiosis where one of the partners is semiautonomous, anabolic and catabolic steps of pathways are located in both the symbiont and the host. In the case of Apicom-

plexa the host apparently does not any longer rely on the former photosynthetic capability of the plastid, but other important biochemical functions may be, at least in parts, located in the "Hohlzylinder". This could include the biosynthesis of fatty acids, amino acids or haem which are known to be located at least in parts in the plastid, and not in host compartments of phototrophic cells. Intracellular coevolution leads to stable networks which cannot be broken when one of the former interests, e.g. photosynthesis, is not sustained any more. Therefore, the living together of two cells may, after initial steps and coevolution, last for ever. Or, after Humphrey Bogart, initiation of cytosymbiosis "is certainly the beginning of a long personal relationship" (Bogart 1944, adapted from the famous movie *Casablanca* after Ingrid was gone).

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## Stomatal Patchiness

By Wolfram Beyschlag and Jürgen Eckstein

### 1. Introduction

"Stomatal patchiness", the phenomenon where the heterogeneous distribution of stomatal apertures results in a patchy carbon assimilation and transpiration, has increasingly drawn the attention of plant ecophysiologists, particularly of those who employ gas-exchange methodology. Since stomatal homogeneity is an assumption in the standard method of calculating net photosynthesis ( $A$ ), transpiration rate ( $E$ ), stomatal conductance ( $G_s$ ) and leaf internal  $\text{CO}_2$  partial pressure ( $c$ ) from gas-exchange measurements (von Caemmerer and Farquhar 1981), stomatal patchiness may lead to erroneous estimations. Although several investigations reported inhomogeneous distributions of stomatal aperture on a leaf surface (e.g. Molisch 1912; Cruiziat et al. 1979; Laisk et al. 1980; Spence 1987; van Gardingen et al. 1989), the consequences on calculated gas-exchange parameters were not assessed. Justification for ignoring inhomogeneous stomatal aperture was suggested by Sharkey et al. (1982), who found (in unstressed leaves) that direct measurements of internal  $\text{CO}_2$  partial pressures were quite similar to values calculated from gas-exchange measurements assuming stomatal homogeneity.

This simplistic picture, however, was complicated by observations that seemingly non-stomatal declines in mesophyll activity in response to stress [decline of carboxylation efficiency ( $CE$ ) and/or maximum photosynthetic capacity ( $A_{\text{max}}$ )] were always accompanied by a similar decline in  $G_s$  (Wong et al. 1979; Tenhunen et al. 1984, 1985; Beyschlag et al. 1987, 1990). In addition, a coupling mechanism between stomatal regulation and mesophyll activity necessary to explain this phenomenon (Wong et al. 1979) could not be found. Nevertheless, the stress hormone abscisic acid (ABA), well known for its important influence on stomatal regulation, seemed to play a major role (Burschka et al. 1985; Raschke and Hedrich 1985), and a breakthrough occurred when stress (and also ABA injection into the xylem of the leaf petiole) was observed to induce localized (patchy) stomatal closure and, in consequence, non-uniform (patchy) photosynthesis within leaves. Thus, "non-stomatal" mesophyll effects related to stress could be explained as resulting from patchy dis-

tribution of stomatal apertures (Downton et al. 1988a,b; Robinson et al. 1988; Terashima et al. 1988; Ward and Drake 1988; Daley et al. 1989; Patzke 1990).

Because of the strong link between "stomatal patchiness" (as the effect became known) and leaf gas exchange, the effect must be considered in the evaluation of gas-exchange measurements. In the simplest case, where patches contain either fully closed ( $x\%$  of leaf surface) or uniformly open stomata, area-specific gas-exchange parameters [e.g. CE and ( $A_{\max}$ )], calculated assuming stomatal homogeneity, apply only to the area of open stomata ( $100 - x\%$ ). Therefore, when the full leaf surface is considered, calculated parameters are underestimated, and use of CE and  $A_{\max}$  as indicators of stress for the activity status of the mesophyll (von Caemmerer and Farquhar 1981) may lead to fallacious interpretations (Fig. 1).

Several recent review papers on stomatal patchiness and related phenomena have summarized much of the relevant information (Terashima 1992; Pospíšilová and Šantrucek 1994, 1997). The present chapter explores stomatal patchiness from the viewpoint of the applied plant ecophysiologicalist interested in understanding situations where estimation of gas-exchange parameters may be biased. To further this understanding,

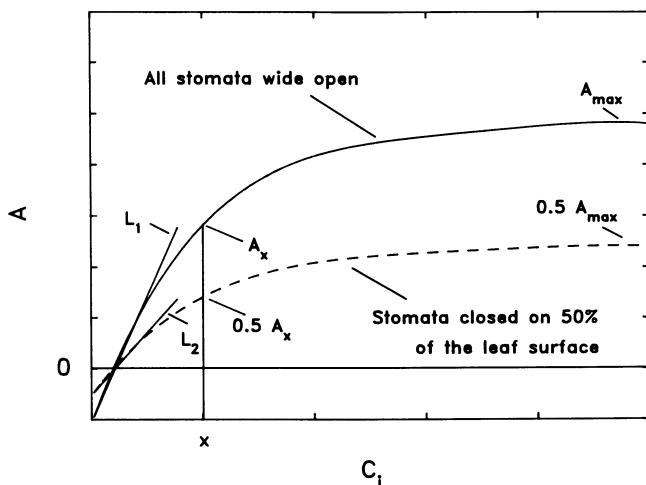


Fig. 1. Theoretical effect of complete stomatal closure in 50% of leaf area of a hypostomatous  $C_3$  plant on the relationship between photosynthesis ( $A$ ) and leaf internal  $CO_2$  partial pressure ( $c_i$ ), with a homogeneous distribution of stomatal aperture. *Solid curve* indicates that all stomata are wide open; *dashed curve* indicates that 50% of the stomata are wide open and 50% are closed.  $CO_2$ -saturated photosynthesis ( $A_{\max}$ ) and assimilation rate at  $c_i$  of level  $x$  ( $A_x$ ) are shown for both situations. Calculated carboxylation efficiency (CE) is the slope of line  $L_1$  when all stomata are wide open, and  $L_2$  when 50% are closed. (Beyschlag et al. 1992)



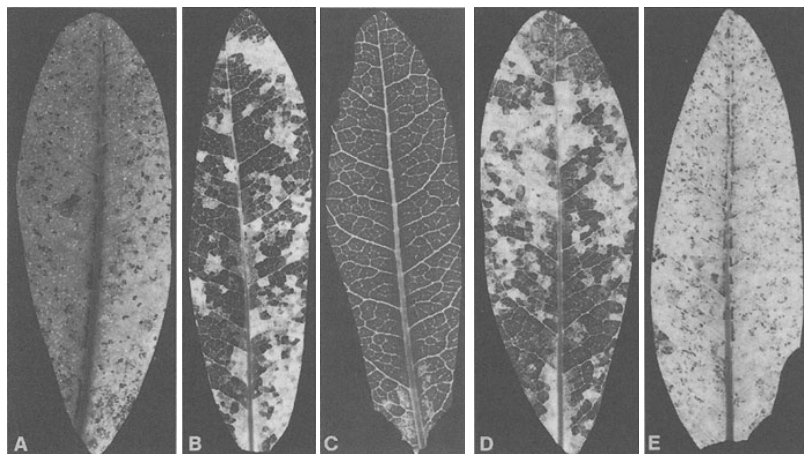
hypotheses related to the mechanisms behind stomatal patchiness are presented.

## 2. What Is Stomatal Patchiness?

The phenomenon of stomatal patchiness is distinguished from random variability in stomatal apertures on the leaf blade (Laisk et al. 1980; Spence 1987) by having clusters of stomata with similar aperture exhibiting similar behaviour (Pospíšilová and Šantrucek 1994, 1997). The spectrum of phenomena considered as stomatal patchiness contains all situations where apertures within regions are autocorrelated, and includes at one extreme nearly bimodal distributions of apertures among areas containing either fully open or essentially fully closed stomata (Beyschlag and Pfanz 1990; Beyschlag et al. 1992).

## 3. How Can Stomatal Patchiness Be Detected and Quantified?

There are direct, semidirect and indirect methods to detect stomatal patchiness. Direct detection can be achieved (1) by direct microscope observation (Omasa et al. 1985; Kappen et al. 1987; Eckstein 1997) and (2) by scanning electron microscopy (SEM) with digital image analysis (van Gardingen et al. 1989). Semidirect detection is possible (3) by mi-



**Fig. 2.** Backlit lower leaf surface of five different *Arbutus unedo* leaves immediately after infiltration of water at different times of day. *Dark areas* indicate non-infiltrated parts; *Light areas* indicate infiltrated parts. Photographs were taken at A 9 a.m., B 10 a.m., C 12 p.m., D 4 p.m. and E 5 p.m. (Beyschlag and Pfanz 1990)

crosscope evaluation of stomatal impressions (Farquhar et al. 1987; Terashima et al. 1988; Ward and Drake 1988; Smith et al. 1989) and (4) by pressure infiltration of liquids into the leaves (Fig. 2) (Beyschlag and Pfanz 1990; Düring and Loveys 1996; Düring and Stoll 1996a,b). Most of the indirect methods measure the inhomogeneity of photosynthetic activity caused by stomatal patchiness. This can be done (5) by " $\text{CO}_2$  assimilation and subsequent autoradiography (Downton et al. 1988a,b; Wise et al. 1991; Gunasekera and Berkowitz 1992; Ni and Pallardy 1992), (6) by analysis of the patterns of starch accumulation (Terashima et al. 1988; Beyschlag et al. 1994) and (7) by gas-exchange measurements combined with video analysis of patterns of chlorophyll fluorescence on the leaf blade (Daley et al. 1989; Patzke 1990; Raschke et al. 1990; Mott 1995; Eckstein et al. 1996; Eckstein 1997). Another indirect method which does not include photosynthesis is (8) the thermal imaging of leaf blades, which reveals spatial differences in transpiration rate (Hashimoto et al. 1984; Omasa 1990).

The pressure-infiltration technique is a rather quick and inexpensive qualitative check whether leaves exhibit stomatal patchiness and can be useful in screening large numbers of leaves in the field. However, this method has drawbacks by being a destructive method [as methods (2), (3), (5) and (6)] and, because the threshold aperture for infiltration depends on the surface tension of the infiltration liquid, stomatal variability below this threshold value cannot be detected. To cover a wide range of possible stomatal apertures, it may be necessary to infiltrate several leaves with liquids differing in surface tension as described by Beyschlag et al. (1992). Indirect methods using differences in photosynthetic activity to indicate stomatal patchiness reveal areas of different stomatal limitation of  $\text{CO}_2$  into a leaf, and have been used in many lab studies (e.g. Daley et al. 1989; Raschke et al. 1990). Care must be used, however, to ensure that factors other than stomatal conductance are not limiting photosynthesis (e.g. localized damage of the photosynthetic apparatus by air pollutants).

Many of the methods to detect stomatal patchiness allow quantification of this variability over the leaf blade through computer-aided analysis of images. Quantitative information on the stomatal variability on the leaf surfaces is particularly important for the correction of photosynthetic parameters calculated from gas-exchange measurements under the assumption of stomatal homogeneity (see Sect. 7).

#### 4. How Widespread Is Stomatal Patchiness?

Stomatal patchiness appears to be a widespread phenomenon and has been detected in 240 species from more than 50 families (Eckstein 1997). The phenomenon seems to be restricted neither to particular systematic

groups, nor to particular morphological or anatomical features of leaves. However, different species seem to show different dynamics of stomatal patchiness, and certain anatomical features seem to promote the phenomenon (see Sect. 5.a). Because of this ubiquity, it is very important to determine whether a particular plant exhibits stomatal patchiness under experimental treatments.

## 5. Under What Circumstances Does Stomatal Patchiness Occur?

### a) Leaf Anatomy

Relationships between the presence of stomatal patchiness and anatomical features of the stomatal apparatus probably are not likely (Eckstein 1997). Nevertheless, there is evidence that under certain conditions (e.g. at low  $G_s$ ) the anatomically based statistical variability in the stomatal aperture on a leaf may be important for inducing stomatal patchiness (Eckstein 1997).

Pneumatic compartmentalization of the leaf interior by leaf veins connecting the upper and lower epidermis (heterobaric leaf anatomy), very common in woody and less common in herbaceous species, seems to promote the occurrence of stomatal patchiness. Inhibition of lateral gas diffusion within the leaf is likely to be the cause (Terashima et al. 1988). In homobaric leaves, evidence exists that a pronounced heterogeneity of stomatal apertures can lead to lateral  $CO_2$ -gradients, and in consequence to heterogeneous photosynthetic activity on the leaf blade (Loreto and Sharkey 1990; Patzke 1990; Eckstein 1997; Beyschlag and Phibbs, unpubl.). Therefore, stomatal patchiness can theoretically occur in any plant leaf, but the potential is higher in heterobaric than in homobaric leaves (Eckstein 1997).

### b) Ambient Conditions

In general, the ambient conditions of leaves seem more important than anatomical properties for the occurrence of stomatal patchiness. Stress factors, particularly those which impose water stress on plants, seem to play a dominant role in patchiness formation. The association of stomatal patchiness with stress is also strongly supported by experimental results for several species where the injection of the stress hormone ABA into the xylem of the leaf petiole caused pronounced stomatal inhomogeneities on the leaf surface (Farquhar et al. 1987; Downton et al. 1988a; Terashima et al. 1988; Ward and Drake 1988; Daley et al. 1989; Patzke 1990; Raschke et al. 1990; Cornic and Ghashghaie 1991; Day et al. 1991;

Beyschlag et al. 1994; Olsson and Leverenz 1994; Genty and Meyer 1995; Hirasawa et al. 1995; Mott 1995).

Several factors which impose water stress have been identified as relevant to the induction of stomatal patchiness. These include: (1) low soil water potential (Hashimoto et al. 1984; Farquhar et al. 1987; Kaiser 1987a,b; Downton et al. 1988b; Cornic et al. 1989; Sharkey and Seemann 1989; Raschke et al. 1990; Stuhlfauth et al. 1990; Scheuermann et al. 1991; Gunasekera and Berkowitz 1992; Ni and Pallardy 1992; Wise et al. 1992; Epron and Dreyer 1993; Lechner 1993), (2) salt stress (Flanagan and Jefferies 1989a,b; Downton et al. 1990; Brugnoli and Lauteri 1991), (3) detachment of roots or leaves (Wise et al. 1991, 1992; Matthews and Omasa 1992; Hirasawa et al. 1995; Eckstein 1997), and (4) low air humidity (Beyschlag et al. 1990, 1992, 1994; Bongi 1990; Loreto and Sharkey 1990; Dai et al. 1992; Düring 1992; Mott et al. 1993; Cardon et al. 1994).

Besides factors related to water stress, other stress factors may be relevant to inducing stomatal patchiness. Stomatal patchiness has been shown in association with (1) chilling (Peisker and Tichá 1991), (2) changes in photosynthetic photon fluence density (PPFD) (Cardon et al. 1994, Genty and Meyer 1995; Bro et al. 1996; Düring and Loveys 1996; Eckstein et al. 1996; Eckstein 1997), (3) the gaseous air pollutants  $O_3$  and  $SO_2$  (Omasa et al. 1981, 1987; Ellenson and Amundson 1982), (4) changes in the  $CO_2$  partial pressure of the ambient air (Xu et al. 1994; Siebke and Weis 1995), and (5) the occurrence of photoinhibition and fungal infections (Sharkey et al. 1990).

Features common to stomatal patchiness as induced by stress have been identified and can be summarized. (1) Stomatal patchiness seems to be a dynamic phenomenon, occurring and disappearing in response to many changing environmental factors, some occurring predictably over the diurnal course of microclimatic variation (e.g. Beyschlag and Pfanz 1990; Beyschlag et al. 1992; see also Fig. 2). The speed of environmental changes seems to be relevant, since slow changes (over several days) do not appear to induce patchiness (Gunasekera and Berkowitz 1992; Matthews and Omasa 1992). (2) Most of the ambient factors listed above relate to patchiness formation through stomatal closure. (3) Stomatal patchiness occurs predominantly but not exclusively at low leaf conductance (Eckstein 1997). (4) Conditions during plant ontogeny seem to play a role. In plants grown under constant environmental conditions, the induction of stomatal patchiness results in predominantly bimodal distributions of stomatal apertures (wide open/fully closed), while plants grown under fluctuating environmental conditions tend to exhibit intermediate patterns of stomata patchiness (Wise et al. 1991, 1992; Pospišilová and Šantrucek 1994, 1997; Hirasawa et al. 1995). (5) Despite considerable knowledge about the above deterministic dependencies, it is not possible in every case to precisely predict the stomatal behaviour of a leaf (Eckstein 1997).

## 6. The Mechanism of Stomatal Patchiness

Integrating features characteristic to stomatal patchiness (some contradictory) into a mechanistic model of the phenomenon is difficult. Building such a framework depends on answering the question: what causes stomata in a particular portion of the leaf surface (often a compartment of a heterobaric leaf) to behave differently from stomata in adjacent areas?

### a) The Role of ABA

The first attempt to explain heterogeneous stomatal behaviour on the leaf blade hypothesized a heterogeneous sensitivity to ABA among stomata within different leaf regions (Mansfield et al. 1990; Schroeder and Hagiwara 1990). However, recent experiments with transgenic plants (Eckstein 1997), where the effect of ABA is blocked, showed clearly that ABA is not essential to induce stomatal patchiness. Thus, studies showing the occurrence of stomatal patchiness following ABA injection into the leaf xylem should be interpreted as ABA lowering  $G_s$  for the entire leaf, resulting in a condition where stomatal patchiness preferably occurs. ABA does not appear to cause heterogeneous stomatal behaviour by itself. Induction of stomatal patchiness as a consequence of any factor causing stomatal closure or reduced  $G_s$  may be explained this way, but does not provide a mechanism for the heterogeneous patches of stomatal aperture.

### b) Water Potential Heterogeneities

Another hypothesis used to explain heterogeneous stomatal behaviour is the assumption that different areas on the leaf blade have different water potentials (Terashima 1992) as found by Rawlins (1963), Hofman and Splinter (1968) and Malone and Tomos (1993). Stomata located close to leaf veins, particularly near large veins, should have a better water supply (thus larger apertures) than stomata located further away from leaf veins (Maier-Maercker 1979; Kappen and Haeger 1991). The kinetics of stomatal movement (particularly rate of opening) also seem affected by this phenomenon (Linsbauer 1917; Raschke 1970).

### c) A Spatial Explicit Model of Stomatal Patchiness

Based on the present understanding of stomatal patchiness and their own experimental results, Haefner et al. (unpubl.) developed a spatially

explicit model for induction of stomatal patchiness based on five assumptions. (1) Stomatal aperture is a linear combination of epidermal and guard cell turgor pressures (Sharpe et al. 1987; Meidner and Edwards 1996). (2) Water flows from the xylem to an evaporating site within the leaf (Nonami et al. 1990; Mott and Parkhurst 1991; Schulze 1994). (3) The evaporating site is in close hydraulic contact with the epidermis (Shackel and Brinckmann 1985; Nonami and Schulze 1989). (4) The osmotic pressure of the guard cell is a function of the water potential (or turgor) of the evaporating site (Cowan 1977; Löscher and Schenk 1978; Schulze 1994). (5) Water moves among water compartments in the leaf in response to water potential gradients.

The occurrence of stomatal patchiness (e.g. subsequent to a decline in air humidity) in a leaf with heterobaric anatomy is explained as follows. Within a particular compartment, some stomata (e.g. those more remote from a leaf vein) close first (assumption 4). Because the epidermis is in close contact with the evaporation sites (assumption 3), a localized increase in epidermal turgor results (see also Sheriff and Meidner 1975). Since stomatal aperture is related to the difference between epidermal and guard cell turgor (assumption 1), the increase in epidermal turgor causes the remaining open stomata of the leaf compartment to close. The water status of the whole compartment improves and the water potential gradient between leaf veins and this compartment decreases (assumption 2). Thus, the water supply increases (assumption 5) in adjacent compartments supplied by the same veins where the stomata are still open (i.e. water potential gradient between the compartment and the veins is still unchanged). This means stomata in these compartments can remain open.

Depending on initial conditions, the model predicts either stable or oscillating patterns among compartments with either open or closed stomata. These predictions are supported by experimental data of Cardon et al. (1994), Eckstein et al. (1996) and Eckstein (1997). To initiate the formation of stomatal patchiness, it is only necessary for some stomata to reduce aperture relative to surrounding stomata. This explains why changes in environmental or physiological conditions which affect stomatal aperture can induce stomatal patchiness. This may be most prevalent at low leaf conductance where the anatomical variability in aperture increases the probability that even small changes in ambient conditions will cause some stomata to close completely, creating pronounced water potential heterogeneity.

#### d) Chaotic Stomatal Behaviour

Another view of stomatal patchiness hypothesizes that chaotic stomatal behaviour may result in the patterns observed (Cardon et al. 1994; Eck-

stein 1997). According to Nicolis and Prigogine (1989) a complex system can become a "dissipative chaotic system" if three conditions are met: (1) energy is supplied from outside the system, (2) there are feedback regulations between several functional parts of the system, and (3) non-linear relations exist between some system parameters. Condition (1) applies to all biological systems. Condition 2 is met because the classical view of stomatal regulation includes several feedback loops (e.g. Raschke 1970). Finally, there are numerous examples of non-linearity within the process of stomatal regulations (condition 3): e.g.  $E$  and  $G_s$  are non-linearly related to each other (Jarvis and McNaughton 1986), and the elastic modulus of the guard cells is not constant which may result in up to three different stable apertures possible for one turgor level (Cowan 1994). Thus, the unpredictability of stomatal behaviour under certain extreme conditions (e.g. high transpiration rates at low water supply; see Eckstein 1997) may be explained by chaotic behaviour.

## 7. Can Gas-Exchange Data Be Corrected for Stomatal Patchiness?

Stomatal patchiness can lead to erroneous estimates of gas-exchange parameters when calculated under the assumption of stomatal homogeneity, particularly in the presence of heterobaric leaf anatomy. Mott (1995) showed the magnitude of error is correlated with degree of heterogeneity in  $c_i$ . In the case of a bimodal distribution of compartments where stomata are either homogeneously wide open or almost closed, it is rather easy to quantify the open and closed fractions of the leaf and correct calculations (for review of equations see Pospíšilová and Šantrucek 1994). Unfortunately, this bimodal aperture distribution seems to be rather rare. In cases with more complex aperture distributions, the bias reduction depends on the resolution of the method quantifying heterogeneity on the leaf. The difficulty in correcting gas-exchange parameters increases with increasing variability among patch means. In some cases it may be very difficult to correct for errors resulting from stomatal patchiness, and gas-exchange methodology may not be useful in realistically quantifying stress effects.

It should be mentioned that the conclusions of Cheeseman (1991), indicating that stomatal patchiness has little effect on  $c_i$  (except in cases of broad aperture distributions), do not apply to classical stomatal patchiness. His use of the term "stomatal patchiness" to describe various normal distributions of stomatal apertures spread rather evenly across the leaf surface is confusing since this is not generally considered stomatal patchiness. The effects on gas-exchange measurements are quite different between his variable distributions spread across the leaf surface and the patchy distributions discussed here. His distributions of stomatal aperture would result in minimal differences in conductance be-

tween leaf compartments, while patchy distributions would result in much more variable conductance between compartments.

## 8. Future Considerations

Contrary to the hypothesis of various authors (Wise et al. 1991, 1992; Gunasekera and Berkowitz 1992; Matthews and Omasa 1992; Martin and Rilling 1993), there is strong experimental evidence that stomatal patchiness is widespread in the field (e.g. Heiland 1994; Keesmann 1995; Düring and Loveys 1996; Eckstein 1997). However, the importance of stomatal patchiness for primary production and water balance of plants growing under field conditions is still largely unknown. To address these issues, information is necessary on whether all leaves of a plant show stomatal patchiness or if this is restricted to certain foliage elements (e.g. sun or shade leaves; see Eckstein 1997). Also, diurnal variations of stomatal patchiness would need to be quantitatively assessed.

While having received some attention, determining whether stomatal patchiness is advantageous to plants is another topic for future research. The hypothesis that non-uniform stomatal aperture distributions increase the water-use efficiency of leaves (Cowan and Troughton 1971; Upadhyaya et al. 1988) has been questioned by Eckstein (1997). Since the E/A-curve approximates an exponential function, the carbon gain and water-use efficiency of a leaf with 50% of the surface containing closed stomata would be less than for a leaf with intermediate but homogeneous stomatal aperture with the same average transpiration rate. Another possible advantage of stomatal patchiness is suggested by Beyschlag et al. (1994) who hypothesized that a periodically changing patchy distribution of stomatal apertures would lower the risk of photoinhibition. Finally, Scheuermann et al. (1991) showed that stomatal patchiness may play a role in the dissipation of radiant energy, because stomatal patchiness in various species predominantly coincided with the occurrence of CO<sub>2</sub> recycling via photorespiration. In contrast, as pointed out by Eckstein (1997), there may be not advantage at all, and stomatal patchiness may be an unavoidable by-product of the rather advantageous (Stahl 1894; Wylie 1943, 1949; Armacost 1944; Canny 1990; Nonami et al. 1990) heterobaric leaf anatomy. Research into this question will have to be conducted under realistic field conditions.

## 9. Conclusions

Stomatal patchiness is:

- A non-normal aperture distribution on the leaf surface.



- A very common phenomenon in many species with various leaf anatomies.
- Not correlated with taxonomic groups.
- Not correlated with the anatomy of the stomatal apparatus.
- A transient dynamic phenomenon which depends on the value and the variability of certain ambient factors (water supply, PPFD, CO<sub>2</sub>, partial pressure).

Stomatal patchiness may be ecologically meaningful because:

- It causes a patchy limitation of photosynthesis (which may lead to errors in the calculation of gas-exchange parameters which may not always be correctable).
- It occurs under field conditions.
- It predominantly occurs under changing ambient conditions (typical field conditions).

Regarding the induction of stomatal patchiness it can be stated that:

- Heterogeneous ABA-sensitivity is not a direct cause.
- Variations in local leaf-water potential are most important.
- Anatomical variability in aperture is important (particularly at low leaf conductance).
- Under certain circumstances, the phenomenon may also be caused by chaotic behaviour of the stomata.

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## Physiology of Poikilohydric Plants

By Wolfram Hartung, Petra Schiller, and Karl-Josef Dietz

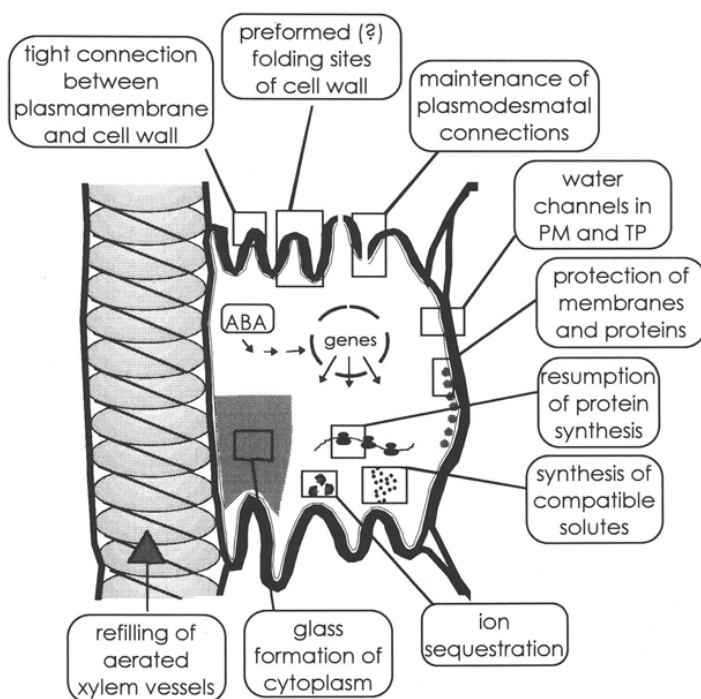
### 1. Introduction

The capability of cells, organs or whole organisms to survive cycles of dehydration and rehydration has evolved in most systematic groups of the plant kingdom. Interestingly, even in the systematic group of the angiosperms, where the sporophytic plant body is usually characterized by high sensitivity towards dehydration, specific structures such as seeds or pollen may undergo excessive water loss without losing viability. Both the distribution of dehydration tolerance throughout the plant kingdom and the occurrence of tolerant structures in most species suggest that many or most structural and metabolic properties required for dehydration tolerance are present in all plants and that only some changes in the developmental program are required to realize the trait of resurrecting a dried plant body. If this provocative conclusion is correct, the question arises why only a limited number of plants have relied on the maintenance of dehydration tolerance. The likely reason is that dehydration tolerance, particularly in higher plants, is advantageous only under very extreme growth conditions but simultaneously poses a severe selective disadvantage in competition for growth, reproduction and spreading under most other growth conditions. In this context, it is important to note that even most resurrection plants must first undergo a period of moderate water loss in order to develop full dehydration tolerance.

Instead of maintaining dehydration tolerance, cormophytes have frequently evolved other mechanisms and structures to avoid the drying out of their plant body, for instance by developing the cuticle as an almost water-impermeable protecting wax layer (Levitt 1980). Such avoidance mechanisms will not be discussed in this chapter. Initially, in fact, poikilohydric plants were defined as organisms devoid of such avoidance mechanisms and, therefore, as plants subjected to rapid equilibration of the plant body with the atmospheric water potential (Walter 1955). In the strict sense, only algae, lichens and some mosses may be assigned to the group of poikilohydric plants. Here, we extend the terminology also to vascular plants, often called resurrection plants, which

survive intensive dehydration and subsequent rehydration and complete their reproductive cycle.

Historically, the study of poikilohydric plants has started with a phase of descriptive wildlife observation which was initiated early in this century (Irmischer 1912; Iljin 1930) and continued until the present day (Gaff 1987; Fischer 1992; Barthlott and Porembski 1996). The record of poikilohydric higher plant species is probably not yet completed. In the second phase which accompanied the first one almost from its beginning, the physiological and biochemical aspects of desiccation tolerance were studied (Bewley 1979). The era of molecular biology of drought and dehydration tolerance began only about 10 years ago. The molecular basis of dehydration tolerance was summarized recently in depth (Ingram and Bartels 1996). However, the genetic approach to an understanding of desiccation tolerance has usually dealt with single, mainly biochemical traits which participate in but cannot explain the phenome-



**Fig. 1.** Factors involved in desiccation tolerance of poikilohydric plants. Factors are either anatomical features like the hypothetical folding sites of cell wall and physiological properties such as refilling of xylem vessels after watering of dehydrated plants or biochemical parameters, for instance synthesis of compatible solutes or of protection proteins. *PM* Plasma membrane; *TP* tonoplast



non of drought tolerance on the whole plant level. This chapter attempts a more general description of the complex traits involved in the expression of desiccation tolerance. Figure 1 summarizes some of the features involved in desiccation tolerance of plants.

## 2. Taxonomic Distribution

Desiccation-tolerant plants have been found in the systematic groups of the thallophytes, i.e. algae, lichens, fungi and mosses, and in various groups of the cormophytes. Table 1 summarizes the major genera of poikilohydric vascular plants. Furthermore, examples of species are given which have been studied in respect of physiological and biochemical aspects. According to Gaff (1977, 1987) most resurrection plants occur in southern and southwestern Africa, southern America, and western Australia. Most dehydration-tolerant vascular plants belong to the group of ferns and species related to ferns, and to the monocotyledons. Especially within the families of the Cyperaceae, Poaceae and Velloziaceae a large number of poikilohydric species are found. On the other hand, no resurrection gymnosperms are known. Even *Welwitschia mirabilis* exhibits only a very weak desiccation tolerance (Gaff 1972).

Most of the dicotyledonous resurrection plants belong to the Scrophulariaceae. Recently, Fischer (1992) has shown that within the Scrophulariaceae 9 of the 10 African *Craterostigma* species are poikilohydric as well as all the 15 African species of the genus *Lindernia*. Among the African Scrophulariaceae the resurrection plant *Craterostigma plantaginum* has been studied most thoroughly so far. This plant has been introduced to research by Prof. O. H. Volk (University of Würzburg, Germany).

*Chamaegigas intrepidus* is a particularly unique member of this group. It is an aquatic resurrection plant that lives in pools on granite outcrops in Namibia. Dehydrated plants survive in depressions for 10–11 months until the rock pools are filled for a few days with rain water. After a few hydration/dehydration cycles, the plants survive in the dried state the longest period of the year, often at temperatures up to 60 °C (Dinter 1918). Figure 2 demonstrates the capability of the resurrection plant *Chamaegigas intrepidus* to survive severe dehydration. In this experiment, photosynthetic quantum yield as calculated from chlorophyll *a* fluorescence parameters was employed as indicator of photosynthetic activity. Upon resupply of water to the desiccated plant, quantum yield rapidly increased from zero to intermediate values and then approached the values observed prior to the desiccation cycle.

Table 1. Taxonomic distribution of poikilohydric vascular plants. Usually a number of poikilohydric species occur in each genus (cf. Gaff 1977, 1987, 1989). Also given is a selection of species that were studied with respect to physiological and biochemical aspects

Taxonomic group	Family	Genus	Example	Reference
Pteridophyta	Actiniopteridaceae	<i>Actiniopteris</i>		
	Adiantaceae	<i>Adiantum</i>		
	Aspleniaceae	<i>Asplenium</i> , <i>Ceterach</i>	<i>C. officinarum</i>	Schwab and Gaff (1986), Schwab and Heber (1984)
	Oleandraceae	<i>Arthropteris</i>		
	Polypodiaceae	<i>Polypodium</i>	<i>P. virginianum</i>	Reynolds and Bewley (1993a,b)
	Schizaceae	<i>Mohria</i>		
	Selaginellaceae	<i>Selaginella</i>	<i>S. lepidophylla</i>	Harten and Eickmeyer (1986), Casper et al. (1993),
	Sinopteridaceae	<i>Cheilanthes</i> , <i>Doryopteris</i> , <i>Pellaea</i>	<i>C. sieberi</i>	Gaff and McGregor (1979)
	Cactaceae	<i>Blossfeldia</i>	<i>B. liliputana</i>	Barthlott and Porembski (1996)
	Gesneriaceae	<i>Boea</i> , <i>Haberlea</i> , <i>Ramonda</i>	<i>B. hygroscopica</i> <i>R. pyrenaria</i>	Navari-Izzo et al. (1994), Sgherri et al. (1994) Gaff and McGregor (1979)
Spermatophyta Dicyledoneae	Myrothamnaceae	<i>Myrothamnus</i>	<i>M. flabellifolia</i>	Gaff and Loveys (1984), Bianchi et al. (1993)
	Scrophulariaceae	<i>Chamaejas</i> , <i>Craterostigma</i> , <i>Ilysanthes</i> , <i>Lindernia</i>	<i>Ch. intrepidus</i> , <i>C. nanum</i> , <i>C. plantagineum</i>	Schiller et al. (1997b), Sherwin et al. (1995) Bartels et al. (1990, 1992), Bianchi et al. (1991), Iturriaga et al. (1992); Furini et al. (1994); Michel et al. (1993); Schneider et al. (1993); Nelson et al. (1994) and others

Monocotyledoneae	Cyperaceae	<i>Afrotrilepis</i> , <i>Carex</i> , <i>Coleochloa</i> , <i>Fimbristilis</i> , <i>Meiriscus</i> , <i>Trilepis</i> <i>Borya</i>	<i>Co. pallidor</i>	Gaff and McGregor (1979)
	Liliaceae		<i>B. nitida</i>	Gaff and Churchill (1976); Gaff and Loveys (1984)
	Poaceae	<i>Brachyachne</i> , <i>Eragrostis</i> , <i>Micraira</i> , <i>Mirochloa</i> , <i>Oropetium</i> , <i>Sporobulus</i> , <i>Tripogon</i> <i>Talbotia</i> , <i>Vellozia</i> , <i>Xerophyta</i>	<i>E. mindensis</i> , <i>S. stapfianus</i> <i>X. scabrida</i>	Gaff (1989); Gaff et al. (1985); Schwab and Gaff (1986); Sutaryono and Gaff (1992) Gaff et al. (1985); Gaff (1989); Sutaryono and Gaff (1992); Kuang et al. (1995) Tuba et al. (1993)
	Velloziaceae			

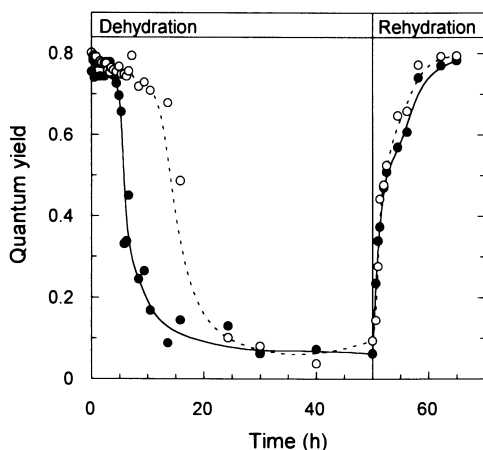


Fig. 2. Quantum yield during a dehydration/rehydration cycle of *Chamaegigas intrepidus*, an aquatic Scrophulariaceae. Either an isolated single plant (closed circle) or a mat of ten plants (open circles) were dried under ambient humidity conditions beginning at 0 h on the time scale. Rehydration was started after 50 h

Gaff (1989) also published a list of 30 poikilohydric grasses, most of them being moderately salt tolerant (up to 250 mM NaCl; Gaff and Wood 1988). Sutaryono and Gaff (1992) showed that at least 15 species, mainly members of the genera *Sporobolus* and *Eragrostis*, have the potential of forage grasses. The digestibility and toxicity of resurrection grasses, the latter tested in a bovine cell system, were comparable with other tropical forage grasses and with wheat. Walter and Volk (1954) and Müller (1985) recommended the poikilohydric grass *Eragrostis nindensis* (syn. *E. denudata*) as a valuable and tasty forage grass which is widely distributed in Namibia and provides a good food supply for cattle, especially in dry years with little precipitation. Productivity of resurrection grasses is low compared with other poikilohydric species, with *Sporobolus* species being five to six times more productive than *Eragrostis* species. Gaff et al. (1985) and Nugent and Gaff (1989) performed electrofusion of protoplasts of poikilohydric and drought-susceptible *Sporobolus* and *Eragrostis* species. However, no regeneration of chimeric plantlets of the fusion products has been published so far. Such chimera may allow us to quantify the genetic complexity of desiccation tolerance.

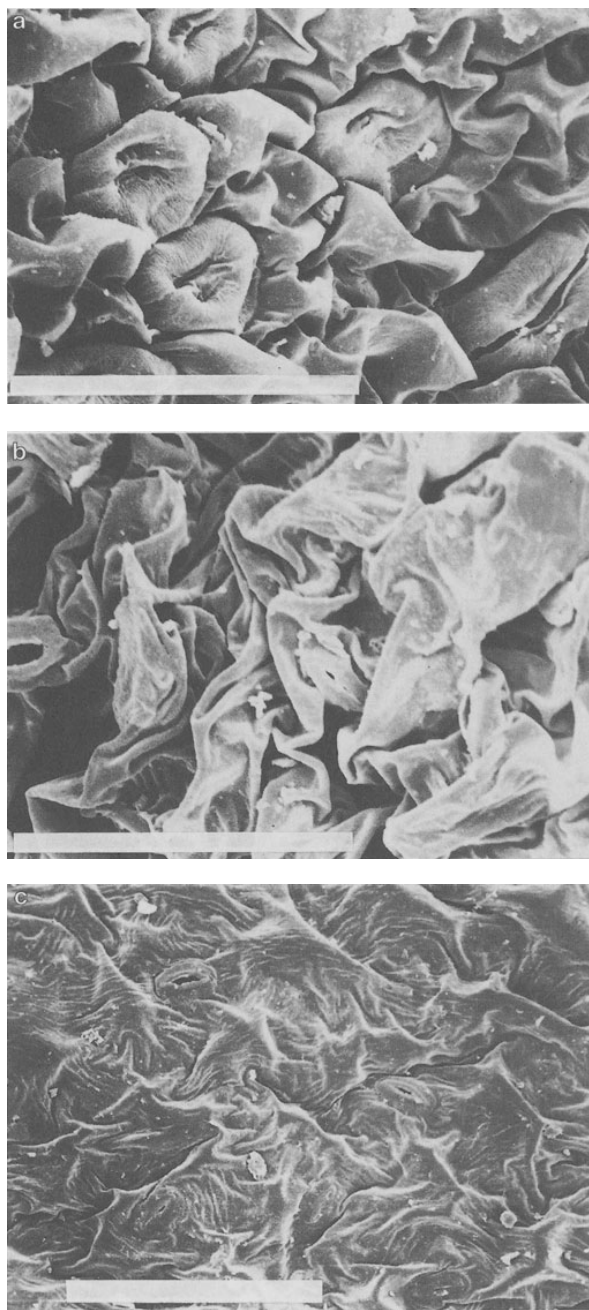
### 3. Structural Features of Resurrection Plants

#### a) Shrinkage of Leaves

There is a major morphological difference between dried leaves of resurrection plants and most drought-sensitive plants. The leaves of resurrection plants shrink and frequently curl up. For instance, the area of *Craterostigma* leaves decreases to less than 15% of that of fully turgid leaves when their water contents drop to about 5%. This is in contrast to tobacco leaves where the area is reduced by less than 50% at a similar extent of water loss. Electronmicroscopic analysis showed that the largely reduced relative size of severely wilted leaves of resurrection plants such as *Ceterach* and *Craterostigma* is accompanied by intense wrinkling of the epidermis in the case of resurrection plants (Fig. 3). A tight connection between the plasma membrane and the cell wall results in a contraction of the whole cell upon desiccation. The phenomenon is called "cytorrhysis". It must be assumed that the extreme shrinkage of drought-tolerant species reflects the capability for cytorrhysis and that this property is essential for the functional conservation of cell structures during severe wilting, for instance the maintenance of the plasmodesmatal connections. In species which perform extensive plasmolysis and no cytorrhysis the plasmodesmatal connections are irreversibly ruptured upon desiccation. Such a severely damaged tissue will not survive rehydration even if other mechanisms provide protoplasmic desiccation tolerance.

#### b) Chloroplasts

During the course of slow dehydration, most poikilohydric monocotyledons lose their leaf chlorophyll. These plants are assigned to the group of poikilochlorophyllous plants which is distinguished from the group of homoiochlorophyllous desiccation-tolerant plants (Gaff 1989). Concomitant with the loss of chlorophyll, the thylakoid membranes and other cellular fine structures are degraded or rearranged. The chloroplasts of the monocotyledon *Xerophyta scabrida* lost their thylakoid membranes and accumulated osmiophilic material which contained carotenoids and lipids (Tuba et al. 1993). Thylakoid membranes began to restructure and chlorophyll was synthesized 10–12 h after the beginning of rehydration of desiccated leaves. The reconstitution of the photosynthetic apparatus was accomplished 72 h after the beginning of rehydration. Photosynthetic activity recovers roughly on the same time scale.

**Fig. 3**

Conversely, poikilohydric mosses, ferns and dicotyledons retain their chlorophyll. This is indicative of a far-going preservation of cell structures. In these homoiochlorophyllous plants, photosynthesis is reestablished more rapidly within a few hours upon rehydration, provided they were hardened either by slow wilting or by addition of abscisic acid (ABA). For instance, this rapid recovery is shown for *Chamaegigas* in Fig. 2 and for *Exormothea* thalli in Hellwege et al. (1994).

### c) Cell Membranes

Rehydration of desiccated tissues leads to leakage of solutes from the cells. The solute loss is not the cause but the indication of damage to the cell membrane (Levitt 1980). Particularly under conditions of severe dehydration, the kinetics and the extent of structural rearrangement of membrane components seem to be important parameters related to desiccation tolerance. When comparing drought-sensitive and drought-tolerant species during hypoosmotic treatment, solute loss was less or insignificant in tissues from resurrection plants and large in drought-sensitive species (Schwab 1986). The molecular basis of the difference in membrane permeability during rehydration is not yet understood. In this context it seems of interest that different from terrestrial non-poikilohydric plants ABA-release to the surrounding alkaline medium was negligible in *Chamaegigas*, probably due to a low permeability of the membranes to ABA (Schiller et al. 1997b). As pointed out in Section 2, *Chamaegigas* grows in pools which are very weakly buffered. Drastic diurnal pH fluctuations occur due to CO<sub>2</sub> consumption and release by photosynthesizing organisms with maximum values above pH10. Therefore, the low permeability of the plasma membrane may be important to avoid loss of ABA to the surrounding medium.

Spickett et al. (1992) have performed <sup>31</sup>P-nuclear magnetic resonance (NMR) studies with dehydrated root tips of maize. During desiccation, they observed an increase in inorganic phosphate and of phosphocholine. Especially the latter was explained by membrane damage due to dehydration. Additionally, a transient alkalization of the root cell cytosol was detected. The authors of this chapter have repeated similar experi-

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Fig. 3. Surface scan of dried leaves by scanning electron microscopy. A Lower epidermis of a *Ceterach officinarum* leaf dried to 4% relative water content; B upper epidermis of a *Craterostigma plantagineum* leaf with 5.4% relative water content; C upper epidermis of a strongly wilted spinach leaf with 9.8% relative water content. The white bars correspond to 50 µm. (Schwab 1986)

ments with intact plants of *Chamaegigas intrepidus*. Even massive dehydration with PEG 600 did not affect the levels of inorganic phosphate, or of phosphocholine. Cytosolic pH remained also stable (Schiller et al. 1997a), indicating that this species does not respond to desiccation as it is known for non-poikilohydric plants and that its membranes seem to be well protected.

#### d) Cell Wall

It was shown as early as 1924 by Heil that cell walls, especially xylem elements of *Chamaegigas*, fold during drying. Cell walls of other resurrection plants such as *Craterostigma nanum* are also folded extensively on drying. The "accordion"-like contraction was possibly responsible for the unusual pressure/volume relation found in these species. As the tissue dried and the vacuoles contracted the cell wall folded, thus preventing excessive tension developing between cell wall and plasmalemma (Sherwin et al. 1995). Large volume changes occurred with little changes of pressure. It appeared that cell wall elasticity changed from being fairly rigid at high relative water contents (RWC) to being elastic at lower RWC. The capability of cell walls for extensive contraction is not only observed in desiccation-tolerant plants but also in specialized cells such as the hydrenchyma cells of desiccation-sensitive *Peperomia* species. There hydrenchyma cells serve as water storage compartments. Upon water loss, the chlorenchyma cells retain their water content at the expense of the hydrenchyma cells which shrink but rapidly expand once the water status of the plants has improved (Schmidt and Kaiser 1987). It has been suggested that the shrinkage of the cells depends on preformed contraction sites in the cell wall.

### 4. Plant Metabolism

Dehydration strongly affects the metabolism of all plants and decreases yield irrespective of the plants' drought tolerance (Lösch 1996). Therefore, large efforts have been made to gather information on the effects of drought on growth and metabolism of mesophytic plants. Conversely, only a limited number of approaches has dealt with the impact of water loss on the metabolism of resurrection plants. However, from the results of both types of studies it appears that many effects of drought-stress on metabolism are equally experienced by dehydration-sensitive species and drought-tolerant species once subjected to a dehydration regime (Gaff 1989).



## a) Photosynthesis During the Dehydration/Rehydration Cycle

Even a slight reduction in leaf relative water contents of higher plants, usually by 5–15%, leads to an inhibition of photosynthetic carbon assimilation. The inhibition of carbon assimilation is accompanied by a drop in transpiration and a decrease in intercellular  $\text{CO}_2$  concentration and, therefore, can be traced back to stomatal closure. It is not caused by an inhibition of photosynthesis, at least not to a major extent (Kaiser 1987; Chaves 1991). In excised leaves, the response is not much different between leaves of mesophytes such as *Spinacia oleracea* or *Primula palinuri* (Dietz and Heber 1983) and leaves of resurrection plants such as *Craterostigma plantagineum* (Fig. 4). Removal or by-passing the gas diffusion barrier of leaves with closed stomates, for instance by peeling off the leaf epidermis, by elevating the external  $\text{CO}_2$  concentration to saturation or by use of thin, non-diffusion-limited leaf slices, show that the photosynthetic capacity of the leaves is not or only little affected at that moderate degree of dehydration. Previously reported indications for non-stomatal inhibition of photosynthesis at low water deficit were based on calculated intercellular  $\text{CO}_2$  concentrations ( $c_i$ ). However, there are two major problems with this approach under conditions of more or less closed stomates, i.e. transpiration rates are low and there may be distinct populations of stomates with different degrees of opening lead-

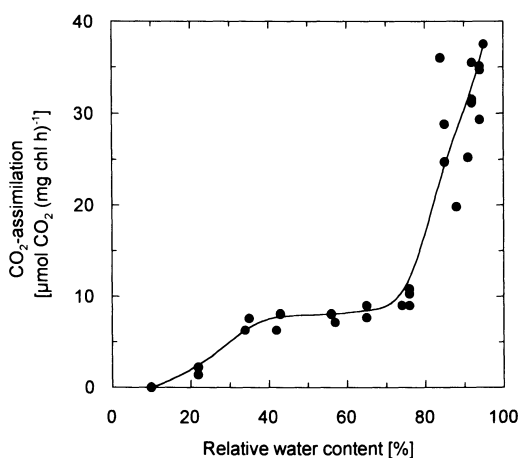


Fig. 4. Photosynthetic  $\text{CO}_2$  fixation as a function of the relative water content of *Craterostigma plantagineum* leaves. Excised leaves were placed in a gas exchange system, dried by passing a gas stream low of water saturation, and photosynthetic  $\text{CO}_2$  fixation was measured as described in Dietz and Heber (1983)

ing to non-uniform gas exchange. In both cases,  $c_i$  may be overestimated, suggesting non-stomatal inhibition of photosynthesis (Downton et al. 1988). The photosynthetic capacity of the mesophyll is usually inhibited at a higher degree of dehydration only. Inhibition of photosynthesis is complete when the water loss approaches 70–80% of the initial relative water content of well irrigated plants (Kaiser 1987; Fig. 4). However, on an extended time scale of days, dehydration of intact potted plants gave a similar profile to inhibition of photosynthesis of excised leaves. Permanent closure of stomates decreased photosynthetic  $\text{CO}_2$  fixation several days after withdrawal of water without affecting mesophyll capacity (Turner and Henson 1989).

However, there also exist clear indications that rapid wilting may be more inhibitory to plant metabolism than slow wilting. Such evidence comes, for instance, from work with drought-sensitive maize, where the breakdown of transport processes between mesophyll and bundle sheath cells was discussed as a likely cause of non-stomatal inhibition of photosynthesis in rapidly wilted leaves. The non-stomatal inhibition was not observed in slowly wilting plants (Saccardy et al. 1996).

Stomatal inhibition of photosynthesis results in a drop in the intercellular  $\text{CO}_2$  concentration to a level close to the compensation point. Under such conditions,  $\text{CO}_2$  cycling between photorespiratory  $\text{CO}_2$  release and photosynthetic  $\text{CO}_2$  fixation allows the maintenance of a considerable light-driven electron transport which protects the photosynthetic machinery from photoinhibition. Chlorophyll *a* fluorescence analysis has shown that the relation between dehydration and loss of photosynthetic activity is similar between drought-tolerant and drought-sensitive species (Schwab and Heber 1984). In fact, in the absence of hardening during a period of slow dehydration, even leaves of most higher resurrection plants and poikilohydric lower plants lose their photosynthetic activity irreversibly. Conversely, upon slow acclimation to lower water potentials, resurrection plants revive and recover more or less to full photosynthetic activity following rewatering. Gas exchange parameters are convenient measures of viability of leaves or tissues. Very fast recovery may occur within minutes, or recovery may be slow and may take some days (Sect. 3.b). The latter is not the consequence of a larger degree of cell destruction. It merely reflects different strategies of organelle maintenance in the dried plant tissue. In contrast to the quite variable recovery of photosynthesis, respiratory  $\text{CO}_2$  release is resumed rapidly and reaches or even surpasses control levels within 30 min (Gaff 1980). Respiration of leaves of *Craterostigma* and other species is restored when their water content approaches about 20% of the initial water content.

The different kinetics in recovery of photosynthesis and respiration in some resurrection species and the differences in restoration kinetics of photosynthesis between species obviously are the function of the struc-

tural preservation of the organelles *and* of desiccation resistance of the protoplasm. The scattered distribution of poikilohydric species throughout the plant kingdom makes it unlikely that the biochemistry of photosynthesis and other metabolic pathways has changed towards increased desiccation tolerance in any case. Results in support of the hypothesis of an essentially unchanged biochemistry were obtained when thylakoid membrane stability was compared between drought-tolerant and drought-sensitive species in the presence of chaotropic ions. Thylakoids lost their activity for cyclic photophosphorylation and for maintaining a large proton gradient in the presence of chaotropic solutes irrespective of the drought-sensitive properties of the source species (Schwab and Heber 1984). Instead of an adaptation on the level of many individual biochemical pathways, restoration of respiratory and photosynthetic gas exchange upon rehydration is indicative of the presence of general and effective drought tolerance mechanisms leading to protection of membranes and macromolecules. The constitutive availability or "just in time" induction of these protective mechanisms are the factors important for an understanding of the physiology of desiccation tolerance.

#### b) Synthesis of Compatible Solutes

One such drought tolerance mechanism leading to protection of membranes and macromolecules is the synthesis and accumulation of low molecular weight organic compounds, for instance sugars, tertiary amines, polyols and amino acids. Loss of water from the cell protoplasm causes chaotropic ions such as  $\text{Cl}^-$  and  $\text{NO}_3^-$  to accumulate. At increasing concentrations, these inorganic anions are potent inhibitors of metabolic functions. In the lower concentration range, the inhibition is reversible since it is mainly based on competition for binding sites. At high cytoplasmic concentrations of anions, as they develop during the phase of severe dehydration, the inhibition may become irreversible by promoting dissociation of protein subunits or denaturation of membranes. The deleterious effects of chaotropic ions are counteracted by so-called compatible solutes. The compatible solutes stabilize proteins and membranes in the presence of the ions which accumulate as the result of increasing water loss, salinity or freezing. In fact, it is a common phenomenon of drought stress that organic compatible solutes accumulate. The chemistry and the amount of accumulated compounds show a species-specific variation (Dietz and Keller 1996). A comparison of drought-sensitive and desiccation-tolerant species revealed higher ratios of sugars to ions in dry leaves of the tolerant species (Schwab and Gaff 1986). During a slow time course of dehydration, sugars are synthesized at the expense of storage carbohydrates. On a dry weight basis, leaves of well-

watered *Eragrostis nindensis* plants contained 5.6% sugars. This figure increased 6.5-fold to 36.3% when the plants were dried slowly. The increase was still significant but less pronounced in *Ceterach* and *Craterostigma* leaves (Schwab and Heber 1984). In addition to sucrose, fructose and glucose, the resurrection plant *Myrothamnus flabellifolia* accumulated glucopyranosyl- $\beta$ -glycerol, trehalose and arbutin (Bianchi et al. 1991).

In fully hydrated leaves of *Craterostigma plantagineum* the overall sugar content was similar to that in dried leaves, but a substantial difference in composition was observed (Bianchi et al. 1991; 1992). The unusual carbohydrate 2-octulose is the dominant sugar in fully hydrated leaves, but upon dehydration it is converted to sucrose. Both carbohydrates represent up to 50% of the dry weight of hydrated leaves or dried leaves, respectively.

Desiccation tolerance of seeds also frequently involves accumulation of sucrose and galactosyl sucrose (Kuo et al. 1988). The stored carbohydrates are metabolized upon germination. Simultaneously, the desiccation tolerance decreases (Blackman et al. 1992). A similar correlation is observed between sugar accumulation and tolerance towards freezing and salt stress. In this light it is not surprising that genes encoding proteins for major steps in pathways leading to osmolyte synthesis have been identified as being induced under drought stress. Examples are glyceraldehyde-3-phosphate dehydrogenase, sucrose-phosphate synthase and betaine aldehyde dehydrogenase (Ingram and Bartels 1996). Compatible solutes have been reported to replace water molecules bound to polar residues of proteins or phospholipids and thereby to prevent denaturation and structural disintegration of macromolecules and membranes (Crowe et al. 1992). An alternative function of sugars such as raffinose, stachyose and other galactosyl-sucrose-oligosaccharides with a higher degree of polymerization is the suppression of crystallization of protoplasmic constituents and the promotion of glass formation at low water content, at least in seed embryos (Bruni and Leopold 1991). In the glassy state a liquid has a very high viscosity. Chemical reactions are slowed down. Residual water molecules are trapped and interactions between cell components are prevented. Therefore, a glassy state is highly stable and ideal to endure anhydrobiosis. Furthermore, upon addition of water, the glassy state melts, and metabolism may be resumed rapidly.

Accumulation of compatible solutes seems to be an important factor involved in the acquisition of desiccation tolerance of the protoplasm; however, it is unlikely to account entirely for any tolerance syndrome. Soybean lines have been identified whose seeds accumulate stachyose and raffinose only to values below 30% of normal lines. These seeds with reduced sugar contents showed unimpaired storage characteristics, i.e.

normal rates of survival of desiccation, and good germination capability (Kerr 1993). On the other hand, the importance of compatible solutes such as mannitol and proline for stress tolerance has been demonstrated in transgenic plants. Tobacco overexpressing pyrroline-5-carboxylate synthetase accumulated 10- to 18-fold increased amounts of proline and was proven to be more tolerant to osmotic stress (Kishor et al. 1995). Expression of the bacterial gene for mannitol synthesis conferred increased stress protection to tobacco (Tarczynski et al. 1993). Both investigations, yet not dealing with resurrection plants, strongly support the conclusion that compatible solutes are also of equal importance for desiccation tolerance.

### c) Detoxification of Products of Oxidative Stress

In addition to increasing concentrations of chaotropic ions, desiccation of cells leads to severe imbalances of metabolism and to an accumulation of toxic metabolites. In photosynthetically active tissues exposed to adverse environmental conditions, absorption of light in excess to the requirements in photosynthetic dark reactions causes liberation of reactive oxygen species which may then oxidize membrane lipids and proteins (Hideg 1996). Formation of superoxide radicals was demonstrated in chloroplasts of drought-stressed wheat using electron paramagnetic resonance (EPR) spectroscopy (Price and Hendry 1991). The suggested mechanism involved stress-induced liberation of catalytic cations, particularly iron and copper, which participate in Fenton-type formation of radicals. Interestingly, superoxide dismutase, glutathione reductase and monodehydroascorbate reductase activities increased under drought stress (Gamble and Burke 1984; Smirnoff and Colom   1988; Jagtap and Bhargava 1995). These data were obtained from work with drought-sensitive species such as barley and wheat. Little information is available for resurrection plants. In the desiccation-tolerant moss *Tortula ruralis* glutathione reductase activity increased during drought stress (Dhindsa 1991). However, again there appears to be no major difference in anti-oxidative defence between drought-sensitive and drought-tolerant species during desiccation. In addition to the inactivation of produced reactive oxygen species, down-regulation of photosynthesis on the level of gene expression, protein amounts and thylakoid structures leads to decreased rates of synthesis of reactive oxygen and contributes to an avoidance of oxidative stress (Ingram and Bartels 1996).

## 5. Absciscic Acid and Induction of Desiccation Hardiness

### a) Absciscic Acid Contents of Poikilohydric Vascular Plants

Although the involvement of ABA in desiccation tolerance of resurrection plants seems to be evident, only little information exists about ABA relations in poikilohydric vascular plants. Table 2 shows the ABA content of leaves of a selection of resurrection plants. A group of plants (*Afrotrilepis pilosa*, *Borya nitida*, *Myrothamnus flabellifolia*, *Vellozia tubiflora* and *Xerophyta dasyrioides*) exhibited a two- to three-fold accumulation of leaf ABA after a period of desiccation. If dehydration of leaves of *Myrothamnus flabellifolia* and *Borya nitida* was too rapid ABA did not increase and leaves did not survive dehydration (Gaff and Loveys 1984).

In some species (*Myrothamnus moschata*, *Sporobolus stapfianus* and *Xerophyta humilis*) ABA accumulated five- to seven-fold. A few more data are available about ABA relations in the poikilohydric grass *Sporobolus stapfianus*. Most distinct ABA increase occurred in the shoots of intact plants when leaves were fully unfolded (approximately ten-fold). In elongation leaves ABA increase was just two-fold. Roots exhibited a small response after dehydration (two- to three-fold increase of internal ABA). Detached organs responded only weakly to desiccation. The authors conclude that ABA does not act as a root-to-shoot stress signal in *S. stapfianus* and that ABA mainly originates from leaves. However, ABA accumulation in leaves can be observed only in intact plants indicating that leaves may receive an ABA signal from other parts of the shoot. Gaff (pers. comm.) observed that a threshold of dehydration (10% water loss) has to be reached to increase ABA formation in *Sporobolus stapfianus*.

Puliga et al. (1996) studied drought effect on the growth of *Sporobolus stapfianus* in relation to xylem sap ABA in comparison with homoiohydric grasses. Under well-watered conditions growth of *S. stapfianus* is approximately 30% of that of common *Festuca* and *Eragrostis* species. Whereas in the latter drought-dependent inhibition of leaf growth was well related to an increase of ABA in the xylem sap, in *S. stapfianus* leaf growth under drought stress was substantially restricted before ABA accumulation occurred. The data indicate different mechanisms of sensing and responding to reduction in soil water availability in drought-sensitive and drought-tolerant species.

Table 2. Abscisic acid (ABA); pmol g<sup>-1</sup> dry wt.) and relative water contents (RWC, %) of hydrated and dehydrated leaves of various resurrection plants

Species	Hydrated			Dehydrated			Maximal change of ABA content (-fold)		References		
	RWC	ABA		RWC	ABA		RWC	ABA			
Dicotyledoneae											
<i>Chamaejasme intrepidus</i> <sup>a</sup>	100	111		72	566	54	1137	35	2145	19.3	cf. Schiller et al. (1997b)
<i>Craterostigma lanceolatum</i>	100	200		74	2040	51	6261			31.3	
<i>Craterostigma plantaginifolium</i> <sup>a</sup>	96	988				51	1738	36	3296	3.3	cf. Schiller et al. (1997b)
<i>Craterostigma plantaginifolium</i> <sup>a</sup>	100	490						9	2800	5.7	Bartels et al. (1990)
<i>Myrothamnus moschata</i>	105	483		73	2523	51	1684	36	642	5.2	
<i>Myrothamnus flabellifolia</i> <sup>a</sup>	100	774				61	1820	32	1681	2.2	Schiller et al. (1997b)
<i>Myrothamnus flabellifolia</i>	97	723								2.5	Gaff and Loveys (1984)
Monocotyledoneae											
<i>Afrotrilepis pilosa</i>	100	54		77	136	50	66			2.5	
<i>Borya nitida</i>	98	325				63	1006			3.0	Gaff and Loveys (1984)
<i>Sporobolus stapfianus</i>	100	15						40	145	6.6	Gaff (personal communication)
<i>Vellozia tubiflora</i>	100	136		77	228	58	145			1.7	
<i>Xerophyta dasyrioides</i>	100	90		81	126	62	297			3.3	
<i>Xerophyta humilis</i> <sup>a</sup>	98	198						40	1254	6.3	Schiller et al. (1997b)

<sup>a</sup> Harvested at the natural site, Namibia. <sup>b</sup> Lab conditions.

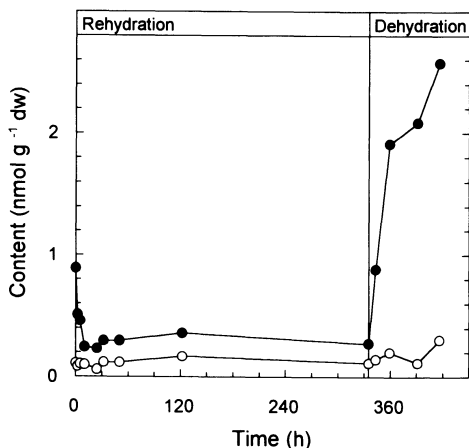


Fig. 5. Abscisic acid content (closed circles) and abscisic acid glucose ester content (open circles) of *Chamaeigigas intrepidus* plants during a time course of rehydration and dehydration

*Craterostigma plantagineum* accumulates ABA three- to six-fold depending on the conditions and the rate of dehydration. Only two other poikilohydric (*Craterostigma lanceolatum*) and the aquatic resurrection plant (*Chamaeigigas intrepidus*) increased their ABA 20–30-fold when desiccated. ABA fluctuations of *Chamaeigigas* during a rehydration/dehydration cycle are shown in Fig. 5.

This may indicate that only a few resurrection plants increase ABA strongly, similar to the poikilohydric liverwort *Exormotheca holstii*, where ABA has also been shown to play an important protective role during desiccation (Hellwege et al. 1994). In the other groups, ABA contents are either higher even under more favourable growth conditions or it may be assumed that a smaller increase is sufficient to initiate hardening.

Different from mesophytic plants, contents of ABA conjugates were high in many resurrection plants. The authors have found ratios of free ABA/conjugated ABA to be 1:2, and in many cases even below 1. This was particularly evident in *Borya nitida*, *Myrothamnus flabellifolia* and *Myrothamnus moschata*. Contents of ABA conjugates may be taken as indication of the stress history of the tissues (Weiler 1980). Each stress cycle stimulates ABA accumulation in the cells, a significant portion of which is conjugated and translocated to the vacuoles. Vacuolar ABA conjugates are not hydrolysed and thus further accumulate with each additional stress cycle.



## b) Beneficial Role of Absciscic Acid During Desiccation

A beneficial role of ABA for dehydrating plant tissues has been observed by Hellwege et al. (1994) with the poikilohydric liverwort *Exormotheca holstii*. Thalli exhibited high ABA concentrations and high desiccation tolerance when growing under natural environmental conditions. After cultivation under well-watered conditions for several weeks ABA amounts were low and desiccation tolerance disappeared unless the non-hardy thalli were treated with ABA. Desiccation-tolerant and ABA-treated thalli exhibited also high amounts of cytosolic desiccation-related proteins as detected earlier in dehydrated corn embryos (Close et al. 1993) and in leaves of *Craterostigma plantagineum* (Bartels et al. 1990).

Werner et al. (1991) performed similar experiments with protonemata of *Funaria hygrometrica*. Again, when pretreated with ABA desiccation tolerance was significantly increased. Even in the highly drought-sensitive submerged living liverwort *Riccia fluitans* ABA increased desiccation resistance significantly (Hellwege et al. 1996). Thalli that were rich in ABA survived a 30% water loss, a drought stress which was lethal for the untreated controls.

According to Bartels et al. (1990) leaves of *Craterostigma* do not need ABA treatment to establish or increase desiccation tolerance. However, when callus cultures were dehydrated cells survived desiccation only when pretreated with ABA.

Gaff (pers. comm.) has investigated the effect of a large number of plant hormones on survival of desiccated leaves of *Sporobolus stapfianus*, including jasmonates, salicylic acid and brassinolides. Only jasmonate, brassinolide, aminocyclopropanecarboxylic acid and ABA improved the desiccation tolerance of suspension cultures of *Sporobolus stapfianus*. Combination of these hormonal factors had neither additive nor synergistic effects.

## 6. Molecular Biology

### a) Synthesis of Proteins

Desiccation tolerance is closely related to protein synthesis during the time course of hardening. In addition, following the phases of (1) hardening, (2) drying and (3) enduring the dry period, resumption of protein synthesis is also particularly important in the (4) phase of rehydration of the tissues. Bewley and coworkers (reviewed in Bewley 1995) have investigated the changes in protein synthesis during desiccation of plant tissues for about 25 years. They have worked on two systems: on the

drought-tolerant moss *Tortula ruralis* and on the seeds of angiosperms. During desiccation and immediately following rehydration of *Tortula* plants, mRNAs were conserved and could be translated in a cell-free system. The pattern of in vitro synthesized proteins was unaltered between hydrated, desiccated and rehydrated mosses. Conversely, the pattern of in vivo labelled polypeptides changed between the treatments (Oliver and Bewley 1984). The difference in protein labelling was interpreted as an indication of a translational level of control which allows the mosses to adapt protein synthesis to the requirements of the fully developed tissue during the dehydration/rehydration cycle. This contrasts with the considerable changes in gene expression observed in seeds during the phases of seed maturation, desiccation and rehydration, and during hardening of desiccation-tolerant higher plants.

In seeds, it is difficult to separate the developmental changes of the embryo from responses involved in the adaptation to desiccation. Examples of genes with high expression during the phase of embryo maturation and acquisition of desiccation tolerance are the so-called late embryogenesis abundant proteins (LEA and LEA-like proteins) initially identified as dominant polypeptides in 2D-gels of maturing seeds (Dure et al. 1981). Six groups of LEA proteins are distinguished on the basis of their amino acid sequences; similarities are particularly intriguing in respect of possible secondary and tertiary structures. They are discussed as water-binding molecules (D 19-group), reverse chaperones (D 113-group) and ion sequestering proteins (D 7-group) (Dure 1993). LEA-like proteins have been identified in vegetative tissues during periods of drought, temperature and osmotic stress.

An example is the adaptation of the liverwort *Riccia fluitans* to adverse growth conditions. *Riccia fluitans* is a fresh water moss and usually lives submerged. Upon transfer onto a dry surface, morphological and physiological changes are induced which also include de novo expression of a LEA-like protein Ric 1 (Hellwege et al. 1996). Strong expression of *Ric 1*-mRNA is also triggered by ABA added to the growth medium of the submerged form. It should be mentioned that *Riccia* does not acquire desiccation tolerance although the ABA-induced changes in expression of *Ric 1* and other genes coincide with increased drought tolerance.

In higher plants, the hardening and desiccation response seem to involve genetic changes in about 20–50 polypeptides of the group of abundant and very abundant proteins. It has to be kept in mind that 2D-gel electrophoretic analyses only account for about 500 polypeptides of the 20 000–30 000 polypeptides present in a typical plant tissue such as leaves (Okamuro and Goldberg 1989). It seems not unlikely that in the vast group of proteins translated from low abundant transcripts the percentage of desiccation-responsive genes or proteins is as high as in the group of abundant and very abundant genes and proteins. If this as-

sumption is correct, 800–3000 genes could be involved in the response of plants to desiccation. Many of these genes would then code for regulatory elements, for instance signal transduction and gene regulation. Recently developed new methodology such as differential display may help to elucidate this somewhat hidden part of drought stress effects and desiccation tolerance.

A detailed 2D-gel electrophoretic analysis was performed in the desiccation-tolerant grass *Sporobolus stapfianus* (Kuang et al. 1995). During a first phase of water loss which led to a relative water content between 85 and 51%, 10 polypeptides appeared and 2 increased in amount. In the second phase of 37–4% relative water content, 15 novel proteins appeared and 2 increased in quantity. Simultaneously, 20 polypeptides decreased or disappeared. *Sporobolus stapfianus* leaves only developed desiccation tolerance when attached to the plant. Detached leaves did not survive desiccation. Interestingly, the changes in polypeptide complements found in drying detached leaves were different from attached leaves in the range of high relative water content and no further significant changes in protein were found at lower relative water content. The wilting-induced increase in ABA content was low in excised and large in attached leaves. A plausible working hypothesis is that detached leaves are incapable of increasing their leaf ABA pool sufficiently to initiate the program of hardening.

A second example is provided by comparing the polypeptide pattern of hardy and non-hardy thalli of the liverwort *Exormotheca holstii*. In the pattern of 400 distinct spots on 2D gels, 31 polypeptides increased or appeared upon hardening of the thalli, and 18 polypeptides decreased or were only detected in non-hardy thalli (Hellwege et al. 1994). Interestingly, antibodies directed against a dehydrin of maize and a desiccation-related protein of *Craterostigma plantagineum* recognized cross-reactive bands in drought-hardened and ABA-treated thalli of *Exormotheca* and were absent in non-hardy thalli. This shows that indeed desiccation tolerance of cormo- and thallophytes is at least partly based on very similar biochemical mechanisms.

## b) Gene Expression

Upon drying of resurrection plants, a set of specific transcripts is induced in leaves as well as in other tissues. Several of them have been cloned as cDNAs. The review of Ingram and Bartels (1996) compiles published data on gene expression in desiccation-tolerant plants with emphasis on the molecular biology of *Craterostigma plantagineum* which serves as a model system to study desiccation tolerance on a molecular level. Most changes in gene expression induced during the period of hardening and drying are also observed when fully hydrated tissues of

the plants are treated with the plant hormone ABA. In fact, ABA substituted for other hardening signals such as mild wilting or elevated salt concentrations (Bartels et al. 1990; Werner and Bopp 1993; Hellwege et al. 1994). Drought hardiness-related changes in gene expression include, for instance, soluble enzymes, membrane transporters and structural proteins. Several of these functions have already been addressed above.

Desiccation-related proteins or dehydrins are cytosolic proteins believed to protect desiccation-sensitive enzymes during dehydration. They constitute the D11 group of the family of the late embryogenesis abundant proteins. Their amount and hence their actual cytosolic concentration may be very high. This indicates that they do not have necessarily a catalytic function but can participate in stoichiometric protection of proteins and other cell structures. Dehydrins are Gly-rich polypeptides which remain soluble at the boiling point of water (Close et al. 1993). The molecular masses of dehydrins are extremely variable between 10 and more than 100 kDa. Two homologues have been isolated from *Craterostigma plantagineum*, namely *Dsp14* and *Dsp16* (Schneider et al. 1993). There is some positive relation between dehydrin accumulation and desiccation tolerance. However, their precise physiological function is still unknown.

A number of drought-induced nuclear gene products are directed to the chloroplast and thought to protect photosynthetic structures from denaturation during severe dehydration (Schneider et al. 1993). In *Craterostigma*, *Dsp 22* was highly homologous to early light-induced proteins known to accumulate in thylakoid membranes particularly after transfer of etiolated leaves to the light (Bartels et al. 1992). Homology of *Dsp 22* to a carotene synthesis-related gene *cbr* (Lers et al. 1991) from the alga *Dunaliella bardawil* is suggestive of a possible role of *Dsp 22* as photo-protectant in the photosynthetic reaction centers and antennae during desiccation.

Aquaporins (water channels or major intrinsic proteins, MIP) have been detected both in the plasmamembrane and the tonoplast of higher plants. Increasing mRNA levels encoding MIPs were observed upon wilting of mesophytic plants such as *Arabidopsis*, pea and barley (Guerrero et al. 1990; Yamaguchi-Shinozaki et al. 1992; Hollenbach and Dietz 1995) and also in the resurrection plant *Craterostigma plantagineum* (Bartels et al. 1996; Ingram and Bartels 1996). Aquaporins are likely to speed up water uptake into desiccated poikilohydric tissues during rehydration.

Increased gene expression under drought conditions or after application of ABA was observed for enzymes involved in the synthesis of protective sugars such as sucrose and trehalose, for proteins of the antioxidative defence (Sect. 4.c), for proteases and for proteins participating in the transfer of cuticular monomers from the protoplast to the site of cuticle assembly at the leaf surface (lipid transfer proteins). A specific

and beneficial function of these and other enzymes and proteins has been demonstrated in plants subjected to drought stress. Their involvement in the expression of desiccation tolerance has still to be proven.

### c) Gene Regulation

Regulation of gene expression involved in metabolic adaptation has been studied at the level of the promoters. Promoter elements responsible for the drought- and ABA-dependent gene activation have been identified and reviewed by Ingram and Bartels (1996). Multiple copies of an ABA-responsive element (ABRE) were fused to a minimal 35S-promoter. This promoter construct allowed the stimulation of reporter gene activity by ABA (Guiltan et al. 1990). Other studies have established that there is also an ABA-independent signal transduction pathway which is involved in the changes of gene expression under drought stress. However, only few genetic studies have dealt with gene regulation in resurrection plants. Michel et al. (1993, 1994) have characterized two promoters of *Craterostigma plantagineum* which respond to drought and ABA. The authors showed that ABA-responsiveness is not dependent on classic ABRE domains. Instead, novel nuclear DNA-binding proteins could be characterized which bind to the promotor. The promotor-element binding proteins could be isolated from callus and leaves only after ABA treatment. Studies on the promotor elements and gene regulation in *Craterostigma* are in progress using heterologous expression of promotor-reporter gene-fusions in *Arabidopsis* (Furini et al. 1996).

Our present understanding of drought-induced changes in gene expression mainly comes from work with drought-sensitive species. It can be assumed that many details of gene regulation are similar between drought-sensitive and drought-tolerant species. Nevertheless, the comparison of resurrection plants with drought-sensitive mesophytes must be advanced in more detail in order to identify the developmental differences.

## 7. Open Questions and Future Research

Protoplasmic desiccation tolerance axiomatically is a central aspect of survival of dehydrated plants. However, another important feature is the rehydration of the dry plant body. Once the water availability in the soil has improved resurrection plants must rehydrate their roots and shoots. Since at a low relative water content of less than 10%, capillary water has been lost from the xylem, resurrection plants must possess effective mechanisms to refill aerated xylem vessels. Root pressure can realize this

function provided the root cells efficiently reestablish their physiology upon rehydration. No or little information seems available on the performance of roots during a dehydration/rehydration cycle. Most of the work with resurrection plants has focused on vegetative shoot tissues.

The genetic complexity of the indispensable traits required for protoplasmic desiccation tolerance still needs to be unravelled. Transformation of drought-tolerant species, such as tobacco, with constructs leading to overexpression of individual gene products has allowed us to identify a considerable number of genes which increase the resistance of the transformants to stress. However, the importance of these genes for desiccation tolerance cannot be demonstrated unequivocally using this approach. Therefore, it is a major step forward that Furini et al. (1994) recently reported successful transformation of the resurrection plant *Craterostigma plantagineum*. This or a similar system of homologous transformation of resurrection plants will allow us to suppress genes of interest by antisense expression and to test for their specific function and importance under conditions of severe dehydration. Using this approach in concert with the heterologous transformation of drought-sensitive species will lead step-by-step to a deeper insight into the phenomenon of desiccation tolerance.

In addition, we need more information of the anatomical requirements for desiccation tolerance. The understanding of the necessary structural features will then allow us to reject or accept the provocative hypothesis raised in the Introduction that all plants have got most or all genetic information to realize desiccation tolerance.

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## To Be or Not to Be – A Question of Plasma Membrane Redox?

By Olaf Döring, Sabine Lüthje, and Michael Böttger

### 1. Introduction

At the beginning of the 1980s scientists working on plasma membrane (PM)-bound oxidoreductase (redox) activities had to go through thorough discussion in order to persuade their colleagues that electron transport at the PM of plants was no mere fiction but reality. Nowadays, the need to prove the sheer existence of the system(s) no longer exists; however, because the PM redox system of intact plants has, up till now, refused detection without electron acceptors that were added to the experimental solution, the most difficult of the old arguments of doubt still lingers on: What might such a system be good for? Today there are some answers to this question (Bienfait and Lüttge 1988; Crane et al. 1991a,b; Navas 1991; Rubinstein 1992; see also the complete issue of *Protoplasma* 184 (1–4), 1995); yet it appears that there are too many answers and no plain way to decide which one is right. In experiments with PM-redox activity an impermeant electron acceptor, e.g. ferricyanide, is almost always added to the experimental solution, and it is therefore not clear what the system would do, if anything, without such a treatment.

It is an established fact that electron transport in the membranes of biological systems is mediated by proteins that are connected through electron transferring compounds, some of which move freely in the lipid bilayer, e.g. quinones. It is not too fantastic to postulate an analogy between electron transport in membranes of mitochondria, chloroplasts or bacteria and electron transport at the PM of plants. As long as there is no information on the internal structure of the PM electron transport, a concept like "cytoplasmic donor oxidase  $\Rightarrow$  low molecular weight electron transporter in or across the membrane  $\Rightarrow$  acceptor reductase" – for plant PM, i.e. three black boxes in a line – might well be a starting point. Today at least the first of the three black boxes can be filled with a number of details, the second one with a few ideas and a little evidence, and the third with much speculation regarding both function as well as its location within the PM.

Easy as it may seem to put forward such an hypothesis, it is misleading to analyse the data at hand with regard to the stated analogy only

and thereby miss other possible explanations or properties of the electron transport system.

Despite the problems we have with assigning a function to an electron transport system in the PM of plants the basic evidence for its existence is surprisingly clear and several effects linked to PM electron transport can be measured quite reliably:

- Many electron acceptors added to the apoplastic solution are reduced at high rates. This reduction can be measured for days and even longer at high rates without a lethal effect on the plant material used. The reduction does not depend on iron deficiency or any other deficiency or stress known. There is no need to induce the "system" as it is present all the time and in all plant material and tissues investigated.

Beside the reduction of the electron acceptor several other effects will always be observed after electron acceptor addition:

- Concomitant with the reduction of the apoplastic acceptor the experimental solution is acidified in addition to the acidification that occurs without external acceptor (Craig and Crane 1981; Ivankina et al. 1984; Rubinstein and Stern 1986). The net acidification observed may exceed the one caused by the PM  $H^+$ -ATPase.
- The PM is depolarized immediately after addition of the acceptor. The depolarization observed was sometimes permanent, sometimes partially transient (cf. Prins and Elzenga 1991; Lüthje et al. 1997).
- A release of  $K^+$  ions was reported after electron acceptor addition (Ivankina and Novak 1988; cf. Bernstein and Dahse 1992).
- Membrane conductance is modified in the presence of electron acceptor (Federicio et al. 1984; Thiel and Tester 1990; Grabov et al. 1993; Grabov and Böttger 1994).
- After application of an electron acceptor, changes in cellular NAD(P)H (Sijmons et al. 1984; Qiu et al. 1985; Krüger and Böttger 1988) and glutathione (GSH) levels were observed (Pattison et al. 1987; Seidenberg et al. 1995).
- The addition of ethanol causes the rate of acceptor reduction to increase. The PM also hyperpolarizes in the presence of ethanol and the net proton extrusion rate rises (Craig and Crane 1981; Böttger and Lüthen 1986; Marrè et al. 1992). The effects of ethanol were said to be caused by the action of cytosolic alcohol dehydrogenase (Krüger and Böttger 1988) causing the supply of the cytosolic electron donor NADH to increase. Other data suggest that ethanol might act on PM  $H^+$ -ATPase via a change of the cytosolic redox state rather than by an increase of proton extrusion by a hypothetical electron transport linked proton pump.
- The cytosolic pH changes after addition of artificial electron acceptors. Unfortunately, it is not clear whether it increases or declines. A

drop of pH was concluded from measurements using fluorescence dyes (Marrè et al. 1988a,b; Trockner and Marrè 1988; Pönitz and Roos 1994), while an increase was observed with  $H^+$ -selective microelectrodes located in the cytosol of *Limnobium stoloniferum* root hairs (Grabov et al. 1993). Similarly, the cytosolic pH of *Egeria* increased after ferricyanide addition as measured by SNARF1 (Seminaphthorhodafluor) fluorescence (L. Kuschel, pers. comm.).

- Preincubation of roots with vitamin K stimulated reduction and membrane depolarization after addition of apoplastic electron acceptor, while dicumarol or warfarin, that are supposed to be vitamin K antagonists, inhibited (Döring et al. 1992a,b; Lühje et al. 1992).
- The reduction is sensitive to hormones (Barr et al. 1984; Böttger and Hilgendorf 1988; Lüthen and Böttger 1988; Lühje and Böttger 1989; Betz et al. 1993; Crane et al. 1995).
- Effectors of signalling pathways such as sphingosin, sterylamine, GTP( $\gamma$ )S and mastoparan did increase apoplastic electron acceptor reduction, while staurosporine was ineffective and ocadaic acid inhibited PM redox (Dharmawardhane et al. 1989; Vera-Estrella et al. 1994b).
- Uptake of various anions was inhibited by external electron acceptors, while anion uptake inhibitors stimulated electron transport to external acceptors. These observations were taken as evidence for a role of PM electron transport in anion uptake ("redox anion pump"; Nespurkova et al. 1989, 1993).
- There is some evidence that a nitrate reductase activity is present at the PM of plants. Inhibition of PM-bound NADH-ferricyanide oxidoreductase activity by immunoglobulin G antibodies raised against soluble nitrate reductase (NR) (Jones and Morel 1988) suggested that nitrate reduction at the PM and NADH-ferricyanide oxidoreductase activity might be linked in some way.
- Under iron deficiency stress dicotyledonous plants and also non-grass monocotyledonous plants show an electron transport system in the PM capable of reducing ferric chelates and also most of the electron acceptors used to investigate the constitutive electron transport system (Bienfait and Lüttge 1988). Because the inducible electron transport system appears to be different from the constitutive electron transport system the reader is referred to other reviews covering this topic (see, e.g., the complete issue of *Plant and Soil* 165, 1994).

All these observations clearly show that "something" happens after addition of an artificial electron acceptor. However, reports on the activity of the electron transport system without an added acceptor are lacking. To make the situation even more complicated, an already mentioned, and compared with the constitutive electron transport system well-known iron-reducing system ("turbo"-system) can be induced in the root of PM of dicotyledonous and non-grass monocotyledonous plants by

iron deficiency (cf. Bienfait 1985; Bienfait and Lüttge 1988; Moog and Brüggemann 1994). Grasses do not have such a system, but they still reduce ferricyanide at the PM, and so do dicotyledonous plants in the presence of sufficient iron.

Regarding the information given so far, we are facing a somewhat curious situation. After more than 15 years of work done on redox phenomena at the plant PM (which some believe to be involved in an existing physiological activity they call "physiological function") we neither know nor have any fact-based hypotheses as to what this activity might be. Still more curious, an increasing number of enzymes and molecules, supposedly components of this activity, have been isolated in recent years. At the moment even work on sequencing and cloning of some of these enzymes is well in progress; even so no one really knows what they are working with. To be a bit more realistic, everyone working on PM redox can – if you bother to ask – tell you at least some of his/her favourite functions, but put  $n$  redox researchers together and you will end up with at least  $n + 1$  distinct hypotheses on the function of PM redox activity. Facing this situation the best the authors can do is to give an overview of the opinions of such an hypothetical gathering. The authors will discuss the possibilities from their point of view.

## 2. Redox Constituents

Recent reviews gave a detailed overview of the redox compounds found in higher plant PM (Møller and Crane 1990; Rubinstein and Luster 1993; Bérczi and Asard 1995; Lüthje et al. 1997), so that we can be brief about the matter at this point. The occurrence of redox constituents in PM may change depending on state of development, tissue (leaves, roots, etc.) or pretreatment with pesticides/herbicides and the like.

From plant PM, flavoproteins such as NAD(P)H:(quinone-acceptor) reductase activities (cf. Buckhout and Luster 1991; Serrano et al. 1995), iron-chelate reductase activities (Holden et al. 1994; Bagnaresi and Pupillo 1995) and an NADH-ferricyanide reductase were isolated to homogeneity (Bérczi et al. 1995). An auxin-sensitive so-called NADH oxidase activity was isolated from soybean hypocotyl (Brightman et al. 1988).

Difference spectra and redox titrations of isolated PM show at least three different b-type cytochromes (cyt), i.e. one *high*-potential cyt b-561 ( $E_0 \approx +150$  mV), which seems to be a common compound in all plant materials investigated; a cyt b<sub>5</sub> ( $E_0 \approx +50$  mV) and a *low*-potential cyt b-557 ( $E_0 \approx -60$  mV) similar to the cytochrome of nitrate reductase (cf. Asard et al. 1994).

In contrast to animal PM, ubiquinone was not detected in the PM of higher plants, while evidence was found for a vitamin K-like substance at least in PM isolated from maize roots (Lüthje 1996; cf. Lüthje et al. 1997)

or soybean hypocotyl (R. Barr, F.L. Crane and M. Böttger, unpubl.). Iron, copper and zinc were found in PM preparations (Møller et al. 1991; Lüthje et al. 1995). Whether these elements are attached specifically to the PM or whether they are bound to proteins is unknown. At least part of the iron is protein-bound. This was concluded from the occurrence of b-type cytochromes and inhibition of PM-bound NADH-dependent oxidoreductase activities by iron chelators like thenoyltrifluoroacetone (Brightman et al. 1988; Serrano et al. 1995).

Ferricyanide as an unspecific electron acceptor can be reduced by all the redox enzymes isolated from plant PM so far, with the exception of the NADH oxidase. As a transmembrane spanning structure was not found for any of these enzymes, it is unclear which of these proteins is involved in constitutive electron transfer by intact plants. The interpretation of all these data becomes more complicated when one regards the fact that in experiments done with isolated PM, NAD(P)H-dependent ferricyanide reductase activities have been identified at both the cytosolic and the apoplastic side (cf. Döring and Lüthje 1996).

### 3. Possible Physiological Functions

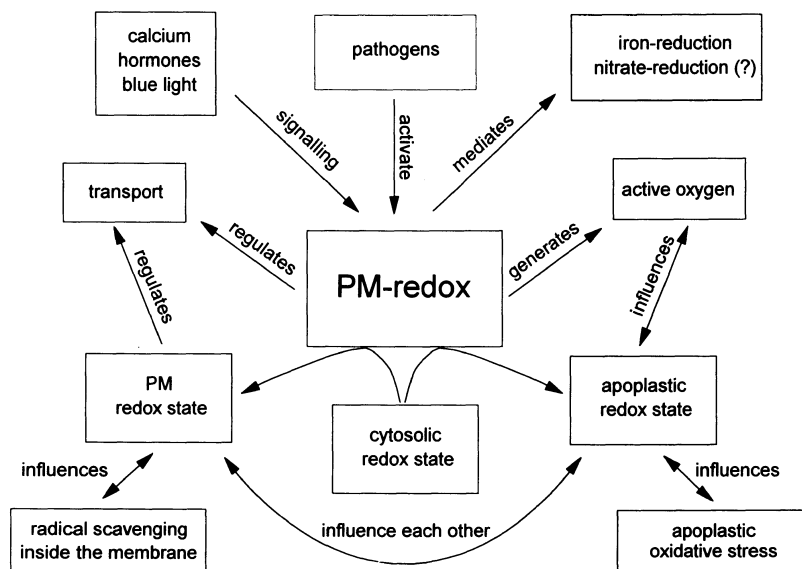
From the observations described in Section 1 and from the enzymic activity and the components found in isolated PM some reasonable speculations on possible roles of PM electron transport can be made. Considering the manifold effects observed *in vivo* after addition of an artificial electron acceptor, it may be possible to conclude that several distinct electron transporting systems are present in the PM, one of them being, for example, the iron-deficiency-induced system. One may also speculate that electron transport across or at the PM affects some very basic regulating or signalling system(s). The physiological activities affected and the substrates found for enzymic activities isolated from PM have led to the proposal of several functions (Fig. 1).

#### a) Transport

##### *α) Proton Pump*

Instead of affecting the PM  $H^+$ -ATPase regulating system (Sects. 3.c, 3.d) the electron transport system itself was proposed to be a proton pump. Although the discussion on electron transport-driven  $H^+$ -extrusion has largely died away, the general argument about the constitutive electron transport system being a proton pump or not is still unresolved (cf. Rubinstein and Stern 1991a; Lüthje et al. 1997).





**Fig. 1.** Proposed physiological functions of constitutive PM electron transport systems. Several proposed physiological roles are summarized in this scheme, although the authors do not think that the multitude of activities can be fulfilled by a single redox system. The large number of functions are discussed in detail in the text

If quinones are involved in PM, electron transport protons will also be translocated. Whether a quinone of the vitamin-K-type is involved in transmembrane electron transfer as proposed by Döring et al. (1992a), and, if that is the case, whether or not protons are released at the apoplastic or cytoplasmic side of the PM, has still to be investigated.

### β) Channels

K<sup>+</sup> transport has repeatedly been found to change after addition of artificial electron acceptors (cf. Lüthje et al. 1997). Inward current increased while outward current decreased. PM K<sup>+</sup>-channels sensitive to depolarization may be the cause for this loss of ions (Hedrich and Schroeder 1989), but the redox state sensitivity of K<sup>+</sup>-channels, as well as dependency on lipid saturation and lipid peroxidation, may also account for the altered ion fluxes (Ganforina and López-Barneo 1991; Lee et al. 1994; Park et al. 1995; Bendahhou and Agnew 1996; Breitweiser 1996). The different shaker K<sup>+</sup>-channel's sensitivity to redox state may vary greatly (Duprat et al. 1995). However, sequence analysis of a β-subunit of a shaker K<sup>+</sup>-channel resembled homologies to NAD(P)H oxidoreductases

indicating that both belong to the same gene superfamily (McCormack and McCormack 1994); thus, perhaps a much closer link between channel gating alteration and PM redox activity might exist.

Other than  $K^+$ -channels are also most likely subject to changing their gating behaviour upon alteration of redox state (membrane/cytosol/apoplast). Reports on in vivo alteration of chloride, nitrate and sulphate transport are available (Nespurkova et al. 1989, 1993) and regulative effects of redox state on cloned  $Cl^-$ -channels in animal systems have been reported (Ran et al. 1992; Ludewig et al. 1996).

While depolarization causes several changes in channel gating (for our discussion not only  $K^+$ -channels but also  $Ca^{2+}$ -channels; see Thuleau et al. 1994), the altered ion in-/efflux may subsequently cause other effects, such as disturbing signal transduction chains and thus causing some of the various effects observed after addition of electron acceptor (Ward et al. 1995).

## b) Ion Uptake

The electrochemical gradient across the PM is considered to be the driving force for uptake processes. Depolarization of the membrane decreases the driving force and thus the potential gradient-dependent uptake diminishes. Not only altered channel gating but also the lower electrochemical gradient may cause a lower uptake of  $K^+$ ,  $Rb^+$  and  $Na^+$  while efflux of these ions increases (cf. Møller and Crane 1990; Lüthje et al. 1997).

Carrier-mediated uptake of amino acids, sugars, nitrate, nitrite, sulphate and organic acid anions also decreased in the presence of apoplastic electron acceptors or SH-group oxidizing reagents (Thom and Maretzki 1985; Rubinstein and Stern 1986; Miklashevich and Novak 1989; Agüera et al. 1990; Nespurkova et al. 1993). It should be emphasized, however, that effects on solute transport caused by establishing heavy electron draw in the apoplast are totally artificial and may not represent any natural process. If the PM electron transport system without added artificial electron acceptor is part of transport regulating system its effect has to be more subtle.

## $\alpha$ ) Nitrate Reductase (EC 1.6.6.1) and Nitrate Uptake

The uptake of nitrate was shown to interact with the constitutive electron transport system in various plants. However, the nature of the interaction is not known yet (Mistrik et al. 1996). There is an ongoing discussion about a nitrate reductase activity at the PM and its involvement in signal transduction for nitrate uptake (Stöhr et al. 1995a; Witt and

Aparicio 1995). Nitrate is taken up by an  $H^+$ -symport (cf. Sivasankar and Oaks 1996; Trueman et al. 1996), which could be inhibited by anti nitrate reductase IgG fragments (Ward et al. 1988). While nitrate uptake seems to interact with the constitutive electron transport system, also the cytosolic NR activity is affected by inhibitors of PM electron transport (Mistrik et al. 1996). Nitrite formed by NR may in turn act on cellular redox state by reaction with ascorbate (Myshkin et al. 1996).

### *β) Iron Uptake/Turbo-Reductase*

Iron is generally taken up by two different strategies: Grasses using an uptake system named strategy II (Römheld and Marschner 1986a,b; Römheld 1987) excrete  $Fe^{3+}$  complexing phytosiderophores. The  $Fe^{3+}$  complexes are reabsorbed by the roots (Bienfait 1985; Longnecker 1988). Non-grass plants import iron as  $Fe^{2+}$  after reduction by an iron-deficiency-inducible trans-PM electron transport system (strategy I; Römheld 1987). Facing iron deficiency these plants enhance PM iron-reducing activity (the turbo-reductase; Bienfait 1985), accompanied by an increase in  $H^+$ -secretion and a release of phenolic compounds capable of reducing/chelating iron (Bienfait et al. 1983; Schmidt 1994). The inducible-iron reduction system differs from the constitutive electron transport system (cf. Moog and Brüggemann 1994; cf. Döring and Luthje 1996). However, a regulatory function in the expression of iron reductase was proposed recently (Schmidt et al. 1996).

### *c) Signalling*

Based on signal transducing pathways in animal systems, similar mechanisms were suggested for plant cells (cf. Yang 1996). Participation of GTP-binding proteins has been proposed in regulation of  $K^+$  channels (cf. Assmann 1996), PM-bound ATPase activity (Qian and Murphy 1993) and in response to plant pathogens and pathogen-attack-related signalling (Legrende et al. 1992; Vera-Estrella et al. 1994a,b; Mehdy et al. 1996). The involvement of PM-bound redox reactions in signal transduction in animal and plant cells has been discussed by Crane (1989). Hormones, calcium and reactive oxygen species are among the cellular messengers (cf. Bush 1995; Kahn and Wilson 1995). In the following (Sects. 3.c.α, 3.c.β and 3.c.γ), we will discuss the possible involvement of redox reaction in cellular signalling and regulation.

### $\alpha$ ) *Hormones*

Besides effects of abscisic acid (Betz et al. 1993; Mistrik et al. 1996) and gibberellic acid (Barr et al. 1984), auxin effects on PM redox activity have been observed by several investigators (Böttger and Hilgendorf 1988; Lüthen and Böttger 1988; Morré et al. 1988, 1995a,b; Morré 1994). The results are contradictory and partially not satisfying because of very low activities involved with even lower hormone effects. Also, secondary processes could not yet be excluded. Thus, from the data at hand a direct involvement of PM electron transport activity in the regulation of elongation growth or other hormone-sensitive pathways could neither be confirmed nor excluded.

Application of the artificial electron acceptors ferricyanide and hexachloroiridate to maize coleoptiles caused a stimulation of elongation growth similar to auxins (Lüthen and Böttger 1993; Carrasco-Luna et al. 1995). As mentioned in Section 1, artificial electron acceptors caused an increase in net-proton secretion, which may induce acid growth. On the other hand these substances might constitute a signal (e.g. reduction of thiol groups at the surface of the PM) that triggers growth (Lüthen and Böttger 1993). This hypothesis is in line with the observation that SH-blockers prevent auxin-induced growth response (Basu and Tuli 1972; Böttger et al. 1984; Spring et al. 1988). In these reactions involvement of the auxin-sensitive NADH oxidase (although postulated recently to reduce SH-groups; Morré et al. 1995b) could not be concluded because the NADH oxidase did not reduce ferricyanide. However, there is some doubt about a general stimulatory function of ferricyanide reductase activity in elongation growth, because a growth inhibition effect was observed for maize roots (Böttger and Lüthen 1986), an effect that parallels the effect of auxin on root growth.

Ascorbate is proposed to regulate peroxidase-dependent polymerization of phenolic compounds and formation of cross-links between soluble molecules of extensin (cf. Vianello and Macri 1991; Arrigoni 1994; Córdoba and González-Reyes 1994). In this case, PM-bound redox activities are only indirectly involved, via a possible semidehydroascorbate reductase that mediates ascorbate regeneration.

This view is in line with the observation that cell differentiation of carrot cells was inhibited after application of ferricyanide (Crane et al. 1984), while ascorbate stimulated this process in onion roots (cf. Córdoba and González-Reyes 1994).

### $\beta$ ) *Blue Light*

Blue light is a signal for the regulation of numerous physiological processes (cf. Ruyters 1984; Budde and Randall 1990; Galland and Senger

1991). PM redox activities are affected by blue light (cf. Rubinstein and Stern 1991b) and there is evidence for a blue light sensor located in the PM (Widell 1987). PM-bound nitrate reductase activity (found in algae) was discussed as a possible photoreceptor, because nitrate uptake and nitrate reduction are stimulated by blue light in a similar manner (Stöhr et al. 1995a; Witt and Aparichio 1995). The algal nitrate reductase activity, a dimer of 95-kDa subunits, is attached to the outer surface of the PM (Stöhr et al. 1995b). Evidence for the occurrence of a similar protein in higher plants, however, is weak (a short summary is given in Döring and Luthje 1996). A blue-light-sensitive cytochrome-flavin complex with characteristic similar to nitrate reductase (i.e. b-type cytochrome, pterin-like substance) has been purified from corn coleoptiles (Leong and Briggs 1981), but there may have been contamination by cytosolic nitrate reductase. Evidence for a possible component of nitrate reductase, pterin, in isolated PM was confirmed recently (Van Gestelen et al. 1996). However, the nitrate reductase activity found in this material was extremely low.

Phosphorylation of a 100–200-kDa protein has been observed in the PM after blue light irradiation for auxin-sensitive plant material but not in roots (Short and Briggs 1990; Reymond et al. 1992a,b; Hager and Brich 1993; Short et al. 1993). Hager et al. (1993) proposed a conformational change of the 100-kDa protein by blue light, a simultaneous redox-dependent activation of a PM-bound protein kinase and subsequent phosphorylation of the 100-kDa protein as a possible mechanism. According to several independent reports, redox-dependent processes appear to be involved in the response of guard cells to blue light (Raghavendra 1990; Pantoja and Willmer 1991; Gautier et al. 1992; Shimazaki et al. 1993).

### *γ) Calcium*

Unspecific effects of calcium,  $\text{Ca}^{2+}$ -chelators and other cations on PM-bound redox activity and the concomitant proton secretion have been reported frequently (Craig and Crane 1981; Rubinstein et al. 1984; Belkoura et al. 1986; Böttger and Hilgendorf 1988).  $\text{Ca}^{2+}$ -calmodulin antagonists and  $\text{Ca}^{2+}$ -chelators modulated PM-bound ferricyanide and NAD(P)H:(quinone-acceptor) oxidoreductase activities in vitro. This suggests a more specific dependence of PM redox systems on  $\text{Ca}^{2+}$  (Belkoura et al. 1986; Böttger et al. 1992; Guerrini et al. 1994). In this context it is interesting that the production of active oxygen species during a respiratory burst (see Sect. 3.e) requires an increase in intracellular calcium (cf. Sutherland 1991).

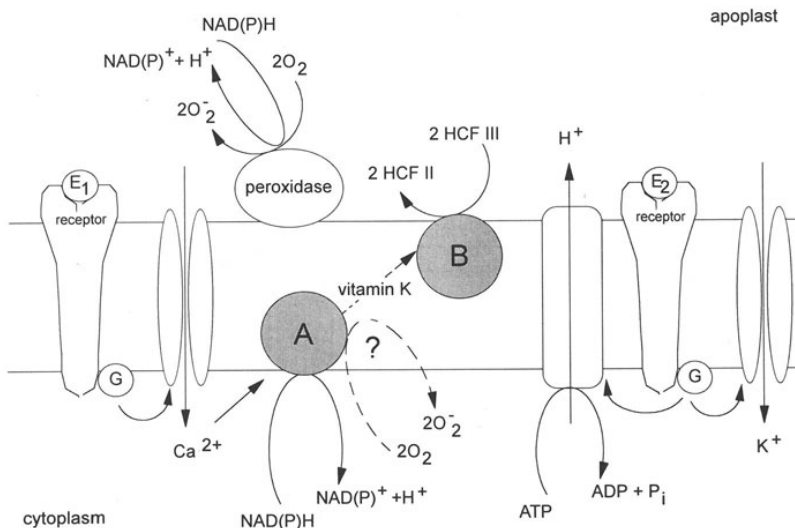
#### d) Cellular Redox State

The redox state of the environment of enzymes influences their conformation and the linking of several enzymes forming bigger structures like, e.g., channels. Thus, enzyme regulation directly or indirectly linked to oxidative stress or redox state has repeatedly been reported for quite distinct activities in animal, plant and bacterial systems (cf. Bienfait and Lüttge 1988; Huppe et al. 1992; Ran et al. 1992; Sakamoto and Tanaka 1992; Youngson et al. 1993; Duprat et al. 1995; Bepalov et al. 1996). The PM  $H^+$ -ATPase depends on a reducing environment, while oxidizing agents block the activity (Elzenga et al. 1989; Qian and Murphy 1993).

#### e) Reactive Oxygen Species

Active oxygen metabolism is among the more prominent choices of a function of PM redox. The concentration of active oxygen species is linked to various physiological reactions; very important among these are pathogen defence, lignification, regulation of membrane transport and herbicide resistance. Both ferricyanide reductase and NAD(P)H-dependent quinone reductase activities increased after treatment with protein-lipopolysaccharide complexes (Valenti et al. 1989; Guerrini et al. 1994). This correlation is perhaps an evidence either for an involvement of the constitutive PM electron transport system in pathogen defence (Fig. 2) or for an involvement in the fast active oxygen production by plant cells observed after elicitor contact (cf. Wingate et al. 1988; Baker and Orlandi 1995; Tenhaken et al. 1995). Subsequent pathogen response reactions involving active oxygen species (second oxidative burst, lignification, apoptosis, acquired resistance, gene regulation) and perhaps enzyme regulation may be also mediated by a PM electron transfer (Vianello and Macri 1991; Bradley et al. 1992; Vera-Estrella et al. 1994a,b; Baker and Orlandi 1995; Mehdy et al. 1996; Watson et al. 1996). While in analogy to the neutrophilic oxidative burst an NADPH oxidase is supposed to be responsible for active oxygen production, the hypothetical involvement of the constitutive PM electron transport system suggests that an NADH oxidase activity could also be the case (Vianello and Macri 1991; Vera-Estrella et al. 1994a,b). Function of peroxidases (EC 1.11.1.7) and SH-groups in generation of the oxidative burst after pathogen attack has been discussed by Bolwell et al. (1995) in detail.

A production of superoxide anion radicals (i.e. a precursor for  $H_2O_2$ ) has been frequently observed at low pH with isolated PM (cf. Vianello and Macri 1991; Qiu and Liang 1995; Qiu et al. 1995; Mehdy et al. 1996). The electron donor of this reaction is NAD(P)H. The physiological significance of this activity is not clear, because the electron donor was



**Fig. 2.** Hypothetical involvement of PM redox in pathogen response. After elicitor  $E_1$  and  $E_2$  binding to one or several distinct receptors a G-protein-(G)-based signal transduction chain is activated. Besides PM  $H^+$ -ATPase,  $K^+$ -channel conductance alteration and increase in  $Ca^{2+}$  influx are mediated. In contrast to animal PM, an NADPH oxidase with similar properties (transmembrane structure, cyt b-245) was not found in plant PM. Aside from hexacyanoferrate III (ferricyanide, *HCF*III) reductase activity, PM-bound NAD(P)H:(quinone-acceptor) reductase (A) are known to be stimulated by  $Ca^{2+}$ . This reductase (or reductase complex) possibly transfers electrons to oxygen and thus  $O_2^-$ . Furthermore, the authors propose that vitamin K (as a mobile electron carrier) mediates an electron transfer to an apoplastic reductase (B) within the PM being under physiological conditions. Under experimental conditions artificial apoplastic electron acceptors (e.g. *HCF* III) can be reduced by this enzyme/enzyme complex. A PM-bound peroxidase is able to generate  $O_2^-$  in the apoplast at expense of NAD(P)H, which in turn may be regenerated by a malate dehydrogenase (cf. Vianello and Macri 1991). Perhaps the  $O_2^-$  generated by the peroxidase and that possibly generated by an NAD(P)H:(quinone-acceptor) reductase (A) serve different purposes. *HCF* II = hexacyanoferrate II, ferrocyanide. Based on models publ. by Mehdy (1994), Knogge (1996) and Kauss and Jeblick (1996)

used in millimolar concentrations. A PM-bound peroxidase activity is most probably involved in this reaction, which could produce  $O_2^-$  in the presence of cysteine or NAD(P)H and oxygen (cf. Vianello and Macri 1991; Bolwell et al. 1995). Inhibition of duroquinone-dependent NADH oxidation at low pH also suggests an involvement of a peroxidase activity (Pupillo et al. 1986).

Duroquinone-dependent NAD(P)H oxidase activity increases in the presence of detergent (Pupillo et al. 1986), which indicated that the binding site of at least one substrate is either located at the cytosolic surface of the PM or becomes accessible after solubilization. However,

generation of  $O_2^-$  by this activity was negligible. On the other hand, an electron transfer chain comprised of several compounds (Fig. 2) will be partially or completely destroyed after solubilization. Furthermore, quinone acceptor reductases showed a distinct reaction with naphthoquinones (Luster and Buckhout 1989; Serrano et al. 1995), and it may therefore be possible that duroquinone acts at a different binding site to vitamin K.

$H_2O_2$  production by young maize roots was not found because release of catalase (EC 1.11.1.6) and peroxidase activities into the apoplast caused the instantaneous destruction of  $H_2O_2$ , if generated (Salguero and Böttger 1995). However, evidence for the production of superoxide anion radicals by intact pea plants was found by Avery'anoff (1985).

As mentioned above, an oxidative burst has been observed after attack by plant pathogens, at least in cell cultures (cf. Sutherland 1991; Boller 1995; Mehdy et al. 1996) and hypocotyl segments (see Kauss and Jeblick 1996). The mechanism behind the oxidative burst of neutrophils, however, seems to be quite different because plants do not have an NADPH oxidase with characteristics comparable with the animal system (cf. Bolwell et al. 1995; Segal 1995). Cytochrome P-450, also discussed as a possible source of  $O_2^-$ , is not found commonly in plant PM (cf. Lüthje et al. 1997). Perhaps cytochrome P-450 is induced under stress conditions (herbicide detoxification; Baerg et al. 1996; Lau and O'Keefe 1996), but as far as the authors' knowledge goes this has not been shown yet for plant PM.

### *$\alpha$ ) Oxidative Stress*

Anyone who is familiar with the modern view of the role of oxygen in life knows that it has become increasingly obvious that oxygen in its different forms can be a very nasty molecule if present in the wrong place. Indeed, organisms undergo much trouble in order to either avoid being oxidized from inside or repair damage already done. Free radicals are detoxified by means of protective systems such as catalase, peroxidases and superoxide dismutase (EC 1.15.1.1), while  $\alpha$ -tocopherol (vitamin E), quinones and ascorbate (vitamin C) act as antioxidants (Larson 1988; Cadenas 1989; Sutherland 1991; Baker and Orlandi 1995; Hammond-Kosack and Jones 1996). In response to oxidative and other stress the activity of oxygen detoxifying enzymes and processes increases.

Oxygen as  $O_2$  is not a very aggressive molecule; other forms of oxygen, in contrast, can be considerably more reactive. The reactivity especially of OH radicals is so high that they react rapidly with many molecules they encounter after generation. If unsaturated lipid acyl chains confront a radical molecule, a chain reaction is initiated converting unsatu-



rated acyl chains into peroxy radicals which in turn cause other acyl chains to be converted.

Tocopherols are located in the lipid bilayer, their chromanol group is close to the hydrophilic part of the membrane (Larson 1988; Gómez-Fernández et al. 1991). Tocopherols partition preferably to the most fluid membrane domains, there forming complexes with lipids containing unsaturated acyl chains (cf. McMurchie and McIntosh 1986; Ortiz et al. 1987; Fukuzawa et al. 1992; Stillwell et al. 1992; Liebler 1993; Kamaleldin and Appelqvist 1996). However,  $\alpha$ -tocopherol itself causes complex changes in membrane fluidity (Stillwell et al. 1992).

In mitochondria ubiquinones are stoichiometrically in excess of respiratory electron chains (Kagan et al. 1994). Due to ubiquinone's biosynthetic pathway it occurs in most of the membranes investigated (Kalén et al. 1987). Besides its function as mobile electron carrier inside the PM of animal system (Crane et al. 1991c), ubiquinol can act either directly as antioxidant or indirectly via a redox interaction with other lipid-soluble antioxidants (cf. Lenaz 1985; Kagan et al. 1994; Constantinescu et al. 1994). In this way a regeneration of tocopherol by ubiquinol is possible. In contrast to animal PM and membranes of several plant organelles (Fryer 1992; Kruk and Strzalka 1995), the authors were unable to find reports on the occurrence of a  $\alpha$ -tocopherol in higher plant PM. Perhaps another molecule with appropriate properties and amount (vitamin K?) could be taken into account to serve a similar function. An electron transfer between mitochondrial cytochrome c and  $\alpha$ -tocopherol has been observed; thus, a protection of membrane components could be mediated by a tocopherol-cytochrome electron transport pathway (Maguire et al. 1992).

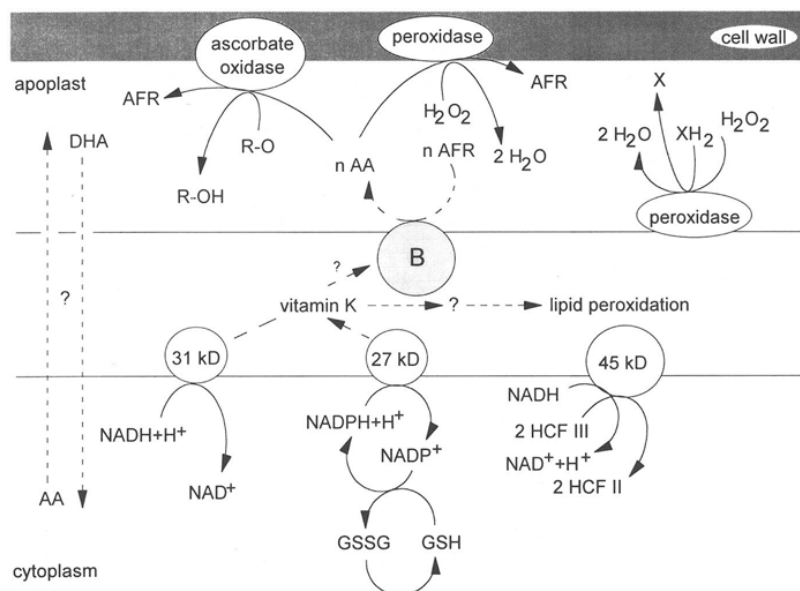
### $\beta$ ) *DT-Diaphorase* (EC 1.6.99.2)

Soluble NAD(P)H:(quinone-acceptor) oxidoreductases (DT-diaphorases) were found in animal and plant cells (cf. Ernster 1987; Valenti et al. 1990). The molecular weight of these proteins is distinct (cf. Lind et al. 1990; Rescigno et al. 1995; Tedeschi et al. 1995; Sparla et al. 1996). The reduction of the quinone is mediated by a two-electron transfer from NAD(P)H to short-chain quinones (e.g. duroquinone, or in artificial systems to menadione). Therefore DT-diaphorase can prevent the generation of active oxygen species by forming semiquinones in a one-electron reduction and subsequent activation of molecular oxygen by the semiquinone (cf. Ernster 1987; O'Brien 1991).

The observation that ubiquinol prevents  $\alpha$ -tocopherol consumption during liposome peroxidation (Cabrini et al. 1991) and the occurrence of an NADH:quinone reductase in animal PM suggests a regeneration of  $\alpha$ -

tocopherol by an ubiquinol and ubiquinone reductase pathway, at least in animal cells (cf. Villalba et al. 1996). However, ubiquinone-10 most likely does not exist in plant PM in significant amounts (cf. L  thje et al. 1997). We propose that vitamin K (a naphthoquinone) could serve this function inside the plant PM (cf. D  ring and L  thje 1996).

The situation is more complicated in plants, because of the occurrence of at least two quinone reducing enzyme activities in the PM. NAD(P)H:(quinone-acceptor) oxidoreductases purified from the PM of higher plants (Luster and Buckhout 1989; Serrano et al. 1995) might be involved in a radical scavenging system, which regulates the redox state of a naphthoquinone pool within the PM (Fig. 3). Reduced vitamin K might act either directly as antioxidant – perhaps with the ability to terminate lipid peroxidation chain reaction – or as regenerator of a hy-



**Fig. 3.** Hypothetical model of function of PM redox activities in radical scavenging. Vitamin K is reduced by NAD(P)H-dependent quinone reductases (27 and 31 kDa). Electrons are either transferred to a reductase (B) located at the apoplastic surface or used to prevent lipid peroxidation inside the PM. A possible electron acceptor of reductase (B) is ascorbate free radical (semidehydro ascorbate, AFR). This reaction regenerates apoplastic ascorbate, used as an electron donor by cell-wall-bound enzymes like ascorbate oxidase or peroxidases. In analogy to animal cells, dehydroascorbate (DHA) generated in the apoplast may be taken up by a carrier so far unknown and reduced by enzymes located in cytosol and possibly by ferricyanide reductase activity (45 kDa) at inner surface of PM. AA ascorbic acid; GSH reduced glutathione; GSSG oxidized glutathione; HCF II hexacyanoferrate II, ferrocyanide; HCF III hexacyanoferrate III, ferricyanide

pothetical  $\alpha$ -tocopherol in the PM (the amount of vitamin K in plant PM may be sufficiently high; Lüthje et al. 1997). The bulk of ferricyanide reductase activity in PM is built up by a dicumarol-sensitive 31-kDa NADH dehydrogenase (Serrano et al. 1995). In contrast to DT-diaphorase, this enzyme does not use NADPH as an electron donor but reduced K-type vitamins at a significant rate.

It is not yet clear if both proteins can serve in radical scavenging. Quinones can react with reduced glutathione (GSH) to form GSH-quinone conjugates (Bellomo et al. 1990). Changes in the cellular GSH/GSSG (oxidized glutathione) ratio and total amounts were observed after application of artificial electron acceptors in vivo (Pattison et al. 1987; Seidenberg et al. 1995). The effect of ferricyanide and other artificial electron acceptors on glutathione levels may be due to a direct or indirect electron flow from the GSH pool to the apoplastic acceptor (Fig. 3). A fall in the GSH level was prevented by regeneration due to a NADP<sup>+</sup>-dependent GSSG reductase.

#### *$\gamma$ ) Ascorbate-Free-Radical Reductase (EC 11.6.5.4)*

The distribution of ascorbate in the living cell makes it a general anti-oxidant (cf. Arrigoni 1994), which could act at both sides of the PM (i.e. cytosol and apoplast). An outline of ascorbate-involving systems in plant cells and the possible function of ascorbate in cellular processes has been discussed in detail by Villalba et al. (1996). Ascorbate interacts with  $\alpha$ -tocopherol similar to ubiquinol (Niki 1987; Buettner 1993) and ascorbate-dependent peroxidases regulate the redox state of the apoplast during oxidative stress (Schmieden and Wild 1994; Polle et al. 1995, 1996; Wise 1995; Córdoba-Pedregosa et al. 1996; Takeuchi et al. 1996). While peroxidases can prevent the formation of free radicals in the apoplast by decomposition of H<sub>2</sub>O<sub>2</sub>, ascorbate scavenges radicals in order to terminate free radical reactions and prevents chain propagation in this compartment (Fig. 3). Thereby, ascorbate free radical (AFR, semidehydroascorbate) is formed by oxidation of ascorbate and further oxidation gives dehydroascorbate, a reaction which is mediated by a cell wall-bound ascorbate oxidase and ascorbate-dependent or other peroxidases (cf. Arrigoni et al. 1981; Penel and Castillo 1991; Villalba et al. 1996). Ascorbate was proposed to be regenerated from semidehydroascorbate by an AFR reductase activity at the apoplastic surface of the PM (Morré et al. 1986). PM-bound b-type cytochromes might be involved in this reaction. Studies with ascorbate preloaded right-side-out vesicles suggest that a reduction of apoplastic AFR by trans-PM electron transport due to the high-potential cytochrome b is possible (Asard et al. 1995). However, chemical reactions due to the standard-redox potential differences of the compounds involved in this reaction,

disproportionation of AFR and subsequent uptake of dehydroascorbate have to be carefully excluded. In vivo investigations point to NAD(P)H as the electron source for trans-PM reactions (Sijmons et al. 1984, Qiu et al. 1985; Krüger and Böttger 1988). Furthermore, NADH-dependent AFR reductase activity was found in animal cells (cf. Villalba et al. 1996). Lipid extraction of animal PM and partially reconstitution with ubiquinones suggest that a quinone may be involved in this electron transfer chain.

However, if cytosolic ascorbate is the natural electron donor for this reaction in plants, AFR will be generated and could react either with soluble AFR reductase activity (Borraccino et al. 1986) or with the PM-bound NADH-dependent ferricyanide reductase purified by Bérczi et al. (1995). The sequence of the latter enzyme closely resembles that of an AFR reductase (I.M. Møller, pers. comm.). This will build up a system for ascorbate regeneration. However, uptake of dehydroascorbate, intracellular reduction either by an AFR reductase or by non-enzymic reactions, and subsequent release of ascorbate is a possible alternative way to regenerate apoplastic ascorbate (cf. Nijus and Kelley 1993; Villalba et al. 1996).

After having read Section 3.e on the generation of active oxygen and on oxidative stress, the reader might ask: Why do these authors propose two contrary and quite important functions for a single system? The authors believe that there is no big difference between transferring electrons to oxygen in order to generate active oxygen and doing the same in an indirect way by giving electrons back to molecules which have been deprived of electrons by oxygen or some radicals. In this way, one system might do two seemingly contrary things that depend on an unknown regulatory mechanism.

#### 4. What Next?

Future research on the constitutive PM electron transport system should focus on several aspects:

- Are the proteins isolated from the PM of some higher plants common compounds of all plant material having the constitutive electron transport system?
- Sequencing of PM-bound redox proteins should show if any of these enzymes has a transmembrane spanning structure.
- While several genes for iron reduction and uptake systems were identified in yeast and also mutants lacking these genes are available for research, reports on the identification of genes for the constitutive electron transport system of higher plants are missing. Up till now, nobody knows if a mutant without the constitutive system is able to exist.

- Do the few not very specific inhibitors of PM redox somehow act on a generation or a scavenging of active oxygen?
- What happens to an oxidative damage of the membrane components in the presence or absence of  $\alpha$ -tocopherol or vitamin K?

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## **Growth Physiology: Approaches to a Spatially and Temporarily Varying Problem**

By Ulrich Schurr

### **1. Importance of Growth for Performance of a Plant in its Environment**

Growing regions are characterized by a high demand for energy and substances. This makes them very susceptible to variations in substance and energy supply, in the short term. In the long term, the distribution and intensity of growth determines the habitus and morphology of a plant and therefore the capacity to acquire nutrients and energy. These features cannot be changed rapidly and the plant has to manage with its habitus even under unfavourable conditions. For example, high nutrient availability favours a high shoot-root ratio, which makes plants more sensitive to drought stress (Schurr and Schulze 1996). Therefore, analysis of growth is paramount to understand the plant's performance in a variable environment.

Plant growth is a complex, multidimensional phenomenon. The three-dimensional structure develops during the growth process in distinct regions of the plant. Within these regions differentiations of cytological, physiological, biochemical and molecular functions take place in parallel. The complexity is further increased by the interactive nature of these processes, making it necessary to approach the analysis of growing regions from different directions simultaneously. This chapter cannot cover the entire field of growth and differentiation, but aims to highlight the importance of spatial and temporal information. The main emphasis will be on the tissue and cellular level; later some biochemical and molecular aspects are discussed. This multiscale approach is needed to understand the processes in growing regions of plants and their interaction with internal and external determinants of growth. It will be necessary to discuss methodological problems in this context, because limitations of techniques hamper in many cases the insight into the spatial and temporal properties of growing zones, but the application of recently developed techniques with high temporal and spatial resolution will result in a more accurate picture of the nature of growth and functional differentiation in plants.

## 2. Organization of Growth at Organ and Tissue Level

Growing regions can be classified into linear, areal and volumetric, which is obviously artificial because growth always results in a three-dimensional corpus of the plant. This classification is mainly based on the arrangement of cell division and cell elongation along the principal direction(s) of growth. Differentiation of cell layers in the direction perpendicular to the main axis of growth is closely linked to these processes (Rost et al. 1988). In this chapter, the author will focus on linear (roots and monocot leaves) and areally (dicot leaves) organized growth due to the almost entire lack of information of sufficient resolution concerning volumetrically growing tissues like fruits or tubers.

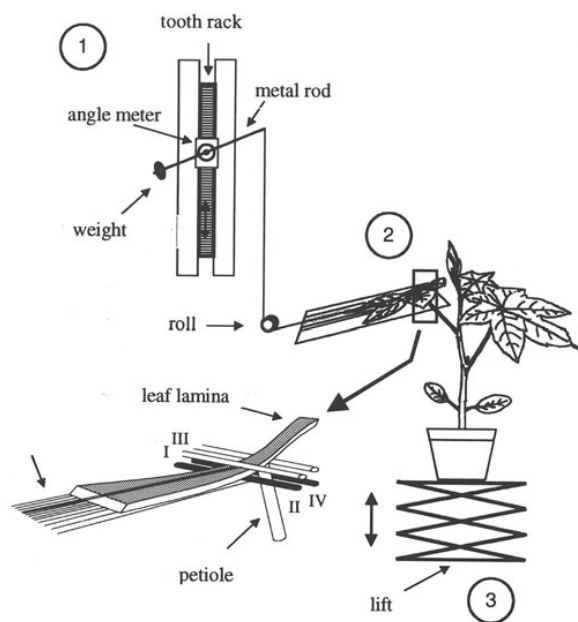
Linear organization of growth and differentiation along a single axis is typical for roots and monocot leaves. In the tip of the root the meristematic zone produces cells, which elongate thereafter in the elongation zone. However, the zones in which these two fundamental growth processes happen are overlapping (MacAdam et al. 1989) and their absolute extension changes during development (Palmer and Davies 1996) and with environmental constraint (Silk 1992). From a physiological point of view, roots and monocot leaves differ in the position of cell division and cell elongation zone relative to the source tissues which supply them with carbohydrates. In the root, the division zone is at the outermost position and has to be supplied through the elongation zone. It has been argued that the differentiation of this supply pathway may be limiting root growth (Bret-Harte and Silk 1994). In monocot leaves, cell division takes place at the leaf base. At least during the initial phases of growth, when the leaf blade is not able to produce adequate amounts of photosynthate to supply the rapidly elongating tissues, the supply of the elongation zone has to happen through the division zone. It has been argued that the cell division zone is also a zone of intermediate storage in order to overcome this problem. Indeed, the analysis of fructan deposition in the growing zone has proven this for carbon (Schnyder et al. 1988). However, the same problem arises with the supply of inorganic nutrients and water (Barlow 1986). This must be considered when deposition rates calculated for inorganic anions in the growing zone of leaves are interpreted (Meiri et al. 1992; Bernstein et al. 1993, 1995). These two examples show nicely how closely physiological differentiation in growing parts of the plant must be linked with the organization of growth.

In dicot leaves, even less is known about the spatial arrangement of growth and physiology. Relatively few studies have focused on this subject since the classical work of Maksymowych on *Xanthium strumarium* (summarized in Maksymowych 1973). A major reason for this are technical and theoretical problems with the analysis of areal growth.



### 3. How to Measure Expansion at Tissue Level with Temporal and Spatial Resolution

One prerequisite for analysing growing tissues is the availability of adequate techniques for the determination of growth. Integrated measures of expansion rate of linear growing tissues can be obtained with various techniques, which differ greatly in their accuracy and the technical effort required (Table 1). The techniques range from measurements with a ruler to continuous determination of leaf length with, e.g., linear variable distance transducers (LVDT) or with angle meters (Heckenberger 1996). In the latter work the elongation of the middle lobe of a leaf of *Ricinus communis* was determined. The leaf base was fixed, while the leaf tip was clipped to a twine and the growth-related movement of the twine was monitored by an angle meter (Fig. 1). This technique can detect length changes down to the range of tens of microns at a high temporal resolu-



**Fig. 1.** Integrated, time-resolved measurement of leaf elongation of *Ricinus communis*. Leaf blade is stretched via nylon thread clipped to leaf tip. 1 Thread is mounted on metal rod, which is connected to angle meter, which gives a continuous signal indicating present angle of its axis. Thread is stretched by a small weight at opposite side of metal rod. 2 Leaf base is fixed by four rods (I and III, transparent; II and IV, aluminium) and leaf blade is located between a network of nylon twines. 3 Leaf is slightly pressed against transparent rods by lifting plant. Height of plant is adapted when petiole has grown. (Heckenberger 1996)

Table 1. Techniques used to analyse growth at tissue level

Technique	Temporal Resolution	Spatial resolution	Apparatus needed	Example reference
Integration over time and space				
Biometric relations	Usually bad	None	Ruler, very accurate calibration needed	
Integration over space				
Continuous length measurement	Very good	None	LVDI or angle measuring device, computer, A-D card	
Continuous area measurement	Good	None	Camera, image analysis	
Space- and time-resolved				
Puncture landmarks	Bad	High spatial resolution increases damage of tissue	ruler	Schnyder et al. (1987); Dodd and Davies (1996)
Particle tracking	High, if direct observation is possible (e.g. roots) In areally growing tissues very time consuming	Dependent on particle density (limited by interference of particles with physiology, e.g. coverage of leaf with particles) Dependent on efficiency of analysis method	Camera, image analysis or ruler	Buff et al. (1987)

Planimetry	Bad, very time consuming, can be enhanced by image analysis	Dependent on efficiency of software Dependent on spatial resolution of camera system Can be very high (see "video image sequence analysis")	Time lapse video or photography Image analysis strongly recommended See "video image sequence analysis" Less complicated analysis, but only recommended in linearly growing tissues	Taylor et al. (1994)  Schmundt et al. (in prep.)
x-t Analysis (Position-time)				
Video image sequence analysis	Can be very high, dependent on: - acquisition rate - efficiency of analysing algorithm (van even be used to obtain online maps of growth)	Can be very high, dependent on: - camera system (optical resolution) - efficiency of analysing algorithm	Time lapse video Image sequence analysis software and appropriate algorithms	Schmundt et al. (in prep.)

AD, analogue-to-digital converted card; LVDT, linear variable distance transducer.

tion (seconds to minutes) when the geometry of the experimental setup is optimized and has been used to follow changes of leaf elongation rate due to variations of turgor pressure in well-watered and water-stressed *Ricinus communis* plants (Heckenberger 1996).

In areally growing tissues, quantitative analysis is often based on biometric relationships, for example the ratio between the product of leaf width and leaf length to the leaf area. This ratio is often constant within one species and can be easily determined (Fig. 2). However, sufficient temporal resolution is hardly obtained in such studies. Additionally, biometric relationships have to be evaluated very carefully in order to obtain correct values, because they change with species and growth conditions and are not constant over the entire growth processes indicated, for example, by an often observed, significant offset of the obtained regression equations (U. Schurr, unpubl. data). Direct estimation of area can be done with commercially available leaf area meters, which determine area from the loss of light flux due to absorption by leaves moved at a constant rate through the light pathway between a light source and a light sensor. Alternatively, simple threshold methods can be used for segmentation of the leaf area on video images or digitized images. Area is then calculated from a calibration of pixel number per area. These techniques usually require detachment of the leaves from the plant. However, in more sophisticated setups, growth can be analysed in attached dicot leaves by video imaging and determination of

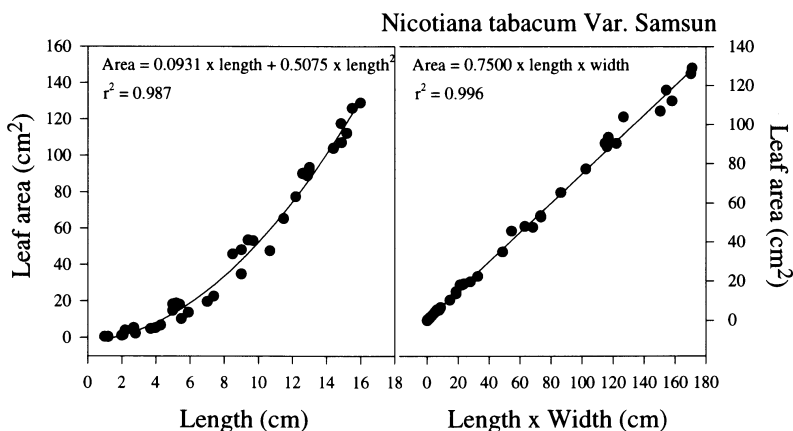


Fig. 2. Biometric relationship between leaf area and (left) leaf length and (right) product of leaf length and leaf width of *Nicotiana tabacum* var. Samsun. Data were obtained by measuring length and width of leaves. Afterwards, outline of each leaf was copied to paper, from which density was determined beforehand by weighing standard areas. Leaf sketches were cut out and their weight was determined on a laboratory balance. In this case, neglectable differences were obtained for regressions forced through the origin and those in which a y-axis offset was allowed. This can be quite different in other species

leaf area by image analysis. The accuracy of such systems is high enough to determine variations of the increase in leaf area, for example in response to air humidity, within minutes (McDonald and Davies 1996).

None of these integrating techniques is able to analyse the nature of the changes in growth rates. Since growth rate of the tissue is the integration of the expansion of individual cells within the growing zone, changes in the growth rate can be caused by variation in cell elongation rates as well as in the number of expanding cells. It has been shown in growing roots that environmental factors can influence expansion rate via either of these mechanisms (Silk 1992). On the other hand, distribution of growth rates can change quite dynamically within minutes (Frensch and Hsiao 1994) and this has functional relevance, for example in gravitropism (Buff et al. 1987). It is therefore obvious that a thorough analysis of growing tissue needs to be based on spatial and temporal information about the growing zones.

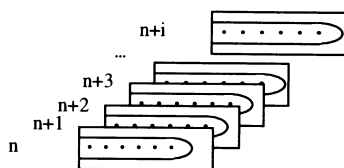
In linearly arranged growing zones, spatial information on expansion rates can be obtained by tracking a series of landmarks along the growth zone. The quality of the landmarks used in such studies ranges from punctures with fine needles (Schnyder et al. 1987; Dodd and Davies 1996) to inert carbon particles (Buff et al. 1987) depending on the accessibility of the growing zone. The expansion rates of the tissue between these landmarks can then either be analysed by determination of the change of distance between the marks after a certain time or, if the growing zone is visually accessible, by continuous observation, e.g. by video imaging.

In areally arranged growth zones it is much more difficult to obtain spatial information. Landmark techniques can be employed in an analogous manner as described above. However, many more measurements are needed to obtain the same spatial resolution. In addition to the increased effort involved in obtaining the basic data sets, growth in an areally growing region is more difficult to analyse for formal reasons. In a linear system all landmarks move in the same direction, i.e. all growth vectors have the same angular component and usually it is easy to determine a distinct point of reference (roots: root tip, leaf: leaf tip) where the integrated elongation rate can be measured. However, in areally growing parts, the data set obtained from landmark distortion is a vector field, in which the individual growth vectors may have quite different values and directions. A good example of such a very tedious work is given by Wolf et al. (1986), in which they determined maps of growth intensities and directions of pathologically misformed leaves of vine. One possibility to circumvent this problem is to determine areal growth rates directly (Maksymowych 1973). This can be done by planimetry of the interveinal areal in time lapse image sequences of leaves (Taylor et al. 1994).

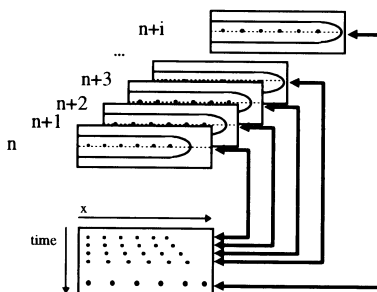
Very few examples of full analysis of growth patterns in areal or even three-dimensional manner can be found in the literature (Gandar 1983a,b; Goodall and Green 1986). A dynamic concept for the description of three-dimensional growth in root apices has been presented (Hejnowicz and Hejnowicz 1991; Hejnowicz and Karczewski 1993). However, these concepts have a rather theoretical character due to the lack of appropriate data sets, because three-dimensional trajectories of cell packets are accessible only in special systems, like roots of *Arabidopsis* (Dolan et al. 1993; Baum and Rost 1996).

Recently, the authors have developed methods that use efficient algorithm for the analysis of image sequences to obtain time-variant maps

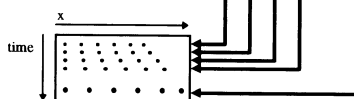
1. Acquisition of image sequence



2. Selection of one line parallel to the major direction of growth



3. Construction of the x-t-plot



4. Analysis of slopes/ calculation of local expansion rates

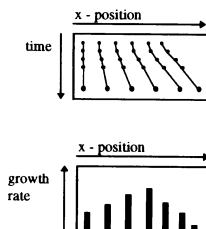


Fig. 3. Schematic construction and analysis of position-time ( $x$ - $t$  plot) from image sequence of a root tip. Landmarks on root tip were obtained in real experiments by application of graphite dust to root surface

of growth rate (Schmundt et al., in prep). In linear growing tissues, spatial and high temporal resolution can easily be obtained with these methods, when so-called  $x'$ - $t$  images (position-time images) are constructed from the digitized image sequence. The principle of this method is illustrated in Fig. 3. Video images are matrices of grey values. For  $x'$ - $t$  analysis, one row or column of the image is selected which is parallel to the major direction of expansion. An  $x'$ - $t$  image is constructed by selecting the same column throughout the entire image sequence and stacking them along the time axis. Distortion of landmarks (e.g. graphite dust) imaged in this column result in streaks in the  $x'$ - $t$  image. The slope of the streaks corresponds to movement velocity and the differentiated values between the streaks can be used to calculate the local expansion rates (Fig. 3; Table 1; Schmundt et al., in prep.) The temporal and spatial resolution is mainly limited by the frequency of image acquisition, the quality of the optical equipment and the algorithms used to estimate growth rates. Such techniques have been used in leaves and roots to follow the diurnal variation of areal expansion rate as well as fast changes of expansion in response to environmental treatments (Schmundt et al., in prep.) Problems arise with this method when the lines of the digitized image are not parallel to the prime growth direction. This occurs, for example, when the tissues bend or move during the experiments. In this case the  $x'$ - $t$  analysis results only in an analysis of the growth component in the direction of the selected column of the image.

More sophisticated methods use the entire image stack of a time series to evaluate the so-called optical flow. These methods can be applied to linear and areally growing tissues to obtain maps of growth rates. The basis of this technique can be illustrated in analogy to the two-dimensional  $x'$ - $t$  images: contrary to the  $x'$ - $t$  plots not only individual lines but the entire images are stacked. Objects present in several consecutive images form continuous streaks in the image stack formed by the time lapse image sequence. Efficient algorithms that have been recently developed to extract local orientation from image stacks have been proven to be applicable to determine the growth vector field in roots and leaves (Schmundt et al., in prep.). Additionally, these techniques deliver localized information on the confidence of the obtained information.

In conclusion, a variety of methods are available now to carry out temporal and spatial analysis of growth on the macroscopic level in linear and areally growing regions of plants. The appropriate method has to be chosen on the basis of the requirements of the experiment for temporal and/or spatial resolution and the availability of equipment. Even though the effort involved in space- and time-resolved studies is much higher than in non-growing tissues it is necessary to use such methods to understand mechanistically the processes in growing tissues.

#### 4. Anatomical and Cytological Aspects of Growing Zones and Kinematics of Growth

Even though this chapter concentrates mainly on expansional growth, spatial aspects of cell division need to be considered. In roots and monocot leaves, cell division is limited to very distinct and small zones. However, the length of the zones showing mitotic activity varies strongly with growth conditions and genotype in leaves (MacAdam et al. 1989) and roots. This means that even in linearly organized growing zones, the actual distribution of cell division and cell elongation zones needs to be determined in order to understand growth mechanistically.

The situation is even more complicated in dicot leaves. In very young dicot leaves, cells undergoing mitosis can be found in the entire leaf. During the development of the leaf, mitotic activity becomes restricted to a small basal area of the leaf prior to full cessation at approximately one-fourth of the end size of the leaf (Maksymowych 1973; Heckenberger et al. 1997). This gradient of growth processes causes significant tip-to-base gradients in leaves of intermediate size, with the cell densities being high at the leaf base and low at the margins of the leaf (Maksymowych 1973; Heckenberger et al. 1997). The decline in mitotic activity is not uniform in the tissue layers. In *Ricinus communis* cell division stops first in the epidermis, while cells are still produced in the palisade parenchyma, and therefore the ratio of, for example, epidermis cells to palisade cells differs between leaves of different size (Heckenberger et al. 1997).

In the cell division zone newly divided cells enlarge again to their initial volume by doubling the cytoplasm. In contrast, in the cell expansion zone, cell enlargement is mainly due to the increase in the vacuolar compartment and cell expansion causes a dramatic decline in cell density. It is easy to imagine that the change in the relative contribution of cellular components per area has consequences for the composition of samples taken for the analysis of composition from growing zones, when, e.g., cell density declines by a factor of 3 within 2 days (Heckenberger et al. 1997). Additional variations result from changes in the ratio between cells of different function (epidermis, palisade, parenchyma).

Two principally different approaches can be used to study the dynamic nature of material changes within the growing zone by kinematic analysis of growth (Silk and Erickson 1979; Silk 1992). In the Eulerian approach (Silk 1992) units of cells and their properties are followed while they move from cell division through cell elongation to the mature part of the tissue. Relative to the root tip or the leaf base, cells are constantly accelerated on their way through the growing zone until they reach the integrated growth velocity, when they appear in the mature zone (Silk 1992; Palmer and Davies 1996). The Eulerian approach is not



only relevant to cytological parameters, but also allows the study of dynamic changes in physiological differentiation within the growing zone (Gandar and Hall 1988).

On the other hand, the Lagrangian approach (Silk 1984) analyses the profiles of properties (cytological, physiological, etc.) along the growing zone. Examples of such "standing profiles" are the distribution of growth rates and of concentrations of carbohydrates (Geigenberger et al. 1996) and inorganic ions (Bernstein et al. 1993) along the axis of growth, which is materially composed of different cells at any time interval. At any time, the parameters distributed along the growing zone are properties of distinct cells that move through these regions.

Recently, these approaches have been intensively used to analyse the impact of environmental factors on growth (for an overview see Silk 1992). The combination of the analysis of integrated leaf growth rate and anatomical records has also been successful in studying the relative impact of drought on cell division and cell elongation (Lecoeur et al. 1995). Analysis of the cellular profiles has proven to be applicable for accurate growth analysis and to allow growth rate profiles as well as growth trajectories to be deduced (Silk et al. 1989). On the basis of such retrospective analysis of position-time relationships of epidermal cells, it has been shown, for example, that cells are only able to start elongating after drought if they were smaller than a certain size at the time of growth cessation (Durand et al. 1995). In this sense, the anatomical pattern of a mature leaf can be regarded as the record of the growth conditions during the time when the cells were contributing to growth. This is not only true for variations in water conditions, but also for nutrient supply during growth (MacAdam et al. 1989). These methods can even be used to study the relative sensitivity of cell division and cell elongation to environmental factors, for which otherwise complex experimental protocols need to be applied (Ranasinghe and Taylor 1996).

The expansion rate of the entire tissue may not depend on each cell layer to the same extent. In cylindrical organs like stems, it has been suggested that the expansion of the epidermis determines the growth rate of the entire plant (Hodick and Kutschera 1992; Kutschera 1992). It is more difficult to imagine how this might be regulated in dorsiventrally flattened leaves, as proposed by Dale (1988). However, the individual cell layers in leaves respond in a highly coordinated manner to environmental stresses like drought, keeping the relation between mesophyll cells and epidermal cells within the developmental pattern. The mechanism behind this is not understood (Heckenberger et al. 1997).

In linear-growing tissues, the spatio-temporal approach has already led to fruitful results in studying the impact of environmental factors. It has been proposed that at least two distinct mechanisms are involved in the control of root and monocot leaf expansion – an accelerating and a

decelerating process (Tomos and Pritchard 1994). The distribution and intensity of these processes determine the length and the intensity of growth and hence the growth rate of the entire tissue. Similar analysis is lacking for areally growing tissues. However, the recent development of macroscopic growth analysis with image sequences will provide the basis for such analysis in the near future.

## 5. Control of Cell Expansion at the Cellular Level

It is well accepted that turgor drives cell expansion. Correlative evidence hints at a role of turgor in controlling growth during the fast responses of growth following rapid changes in the hydraulic status of the growing zone (Pardossi et al. 1994). However, turgor does not control elongation rate during steady state growth. Turgor profiles along the growing zone do not vary with the spatial variation of growth rate and changes in the growth rate are not accompanied by corresponding differences in cell turgor of expanding cells of monocot (e.g. Tomos and Pritchard 1994) and dicot (e.g. Palmer et al. 1996) leaves. This could have even been proposed from the fact that most differentiated cells develop characteristic forms and shapes different from isodiametric cells during growth. A force acting homogeneously in all spatial directions, like pressure, cannot sufficiently explain vectorial expansion growth. Therefore, the controlling mechanisms must be located in the expanding cell walls.

Several studies have revealed a correspondence between potential cell wall loosening components and the spatial distribution of growth rate in root tissues (Pritchard et al. 1993; Wu et al. 1993), hypocotyls (Cosgrove and Durachko 1994; Potter and Fry 1994) and monocot leaves (Palmer and Davies 1996). The few experiments for dicot leaves were either done in cell culture (Potter and Fry 1994) or do not provide spatial resolution (Taylor et al. 1994). The reason for this is mainly that the determination of areal growth rates requires much more efficient methods than in linear growing systems (see Sect. 3). Cell wall loosening compounds change the rheological properties of the cell walls (McQueen-Mason 1995). Several such compounds have been studied intensively during recent years.

Xyloglucan endotransglycosylase (XET) has been proposed to be involved in growth regulation (Fry et al. 1992) on the basis that it can cleave xyloglucan-moieties interlinking cellulose fibres (Carpita and Gibaut 1993). Distribution of XET activity within the growth zone has been found to be closely correlated with growth rate (Wu et al. 1994; Palmer and Davies 1996). Genes encoding for XET have been identified and found to be up-regulated in response to environmental stimuli that affect growth (Xu et al. 1995, 1996). However, isolated XET activity seems to be neither sufficient nor essential for cell wall loosening in *in vitro* extensions assays (McQueen-Mason et al. 1993). Protein fractions

from growing cell walls promoting extension of isolated cell walls did not contain XET activity and the fraction with high XET activity did not induce extension of isolated cell walls.

Expansins induce wall-extension in reconstitution assays with isolated cell walls in vitro (McQueen-Mason et al. 1992). Expansins seem to be involved in acid-induced growth promotion (McQueen-Mason and Cosgrove 1995) and their distribution at least in hypocotyls corresponds well with the local growth rates (McQueen-Mason 1995). Two proteins with apparent molecular masses of 29 and 30 kDa with expansin activity have been purified exhibiting wall-loosening activity without hydolytic breakdown of cell wall components from cucumber hypocotyls (McQueen-Mason et al. 1992). Expansin activity is also abundant in dicot leaves and isolated proteins cross-react with antibodies against the cucumber expansins (Keller and Cosgrove 1995). Expansin activity and amount increased relative to controls in root tips, maintaining growth at low water potential, indicating a role in the adaptation of growth processes to environmental impact (Wu et al. 1996). The extensibility of isolated cell walls and the sensitivity of these cell walls to expansin activity were increased in the apical 5 mm of root tip, while the opposite happened at 5–10 mm behind the root tip, corresponding well to the determined variation in growth profile.

However, the correlation of cell wall loosening activity with expansion rate does not hold in all cases. Palmer and Davies (1996) studied the distribution of XET activity in the growing zone of *Zea mays* leaves in conjunction with the distribution of the growth rates. XET activity peaked shortly before the maximum growth rate was obtained. However, XET activity was still present at the leaf base of fully mature leaves, which did not grow any more. A similar situation can be found with expansin distribution (McQueen-Mason 1995). It may well be that extractable wall-loosening activities become trapped in the cell wall during differentiation. The loss of efficiency might then be due to loss of suitable substrate or stiffening of the cell wall via other mechanisms. Such results emphasize the need to look for growth decelerating processes in growing cells in order to find the regulating mechanisms responsible for the extension of the growth zones in expanding tissues (Tomos and Pritchard 1994).

## 6. Spatial Differentiation at the Cellular Level

Expansion of cells in the growing zone is not confined to being parallel to the prime direction of expansion. For example, epidermal cells expand mainly along the axes parallel to the leaf surface; at the same time the palisade cells elongate in a direction perpendicular to the leaf surface. In order to obtain a vectorial orientation of elongation, cell wall

expanding processes (1) need to be distributed unequally at the cell surface, and (2) have to be highly dynamic during rapid responses to, e.g., environmental impact. This is true for wall-loosening processes and for cell wall synthesis. Very little is known about the temporal aspects of wall-loosening processes and their localization at the cellular level. However, cell elongation rapidly ceases if no additional cell wall material is incorporated.

For example, cortical microtubules are thought to be responsible for the localization of these processes. Recently, technical advances have made it possible to study the dynamics of cortical microtubules in living cells within growing tissues (Yuan et al. 1995; Hepler and Hush 1996). These studies have proven that reorganization of the cortical microtubular structures can occur within minutes (Wymer and Lloyd 1996) and is therefore sufficiently dynamic to explain fast growth variations. Additionally, good agreement was found between the local directions of growth and the orientation of cortical microtubules within the different tissue layers (Yuan et al. 1995).

Obviously the nature of the processes controlling cellular expansion (cell wall loosening, production and deposition of cell wall material) are dynamic. These examples stress the importance of studying cellular growth processes with techniques that allow a similarly relative spatial and temporal resolution as in whole tissue analysis.

## 7. Interactions of Growth, Differentiation and Physiology

Many experiments have studied the change in physiological properties in growing tissues with time. Few have considered the spatio-temporal pattern of growth and the dynamic nature of these tissues (Silk 1984). By combination of published data on the distribution of invertase activity along the root axis and growth rate distribution, Gandar and Hall (1988) have calculated that invertase activity associated with a distinct group of cells rises within 3 h by a factor of 5 after the cells have left the cell division zone of *Zea mays*. Thereafter it drops again in the same cells by a factor of 5 during the following 6 h. This can easily be seen from this type of data by which molecular, biochemical and physiological inventories of cells change within a growing tissue at usual growth rates.

Changes in physiological functions need to be related to the localized function within the growing region. The combination of a kinematic approach with the analysis of spatial distribution of substances within the growing zone can be used to calculate the actual deposition rates of organic (e.g. Bret-Harte and Silk 1994; Schnyder et al. 1988) as well as inorganic (e.g. Meiri et al. 1992) substances. In contrast to non-growing tissues, the deposition rate in growing tissues is significantly different from the change in concentration, because growth dilution as well as the

convectonal displacement of material have to be considered (Silk 1992). Accuracy of the determination of the substance distribution within the usually small growing zones is as important for this kind of approach as the accurate analysis of growth rate distribution. This makes such studies quite tedious, because relatively large numbers of small samples need to be measured. However, recent advances in sampling methods (Tomos et al. 1994) and the development of fast analytical techniques of small samples (Tomos et al. 1994; Bazzanella et al. 1997) will help to overcome these limitations. A promising alternative approach is to use non-destructive techniques, which allow the imaging of physiological, biochemical or molecular properties. Examples of such techniques are the detection of ion distribution by ratio imaging (Hoffmann and Kosegarten 1995; Mühling and Sattelmacher 1995), nuclear magnetic resonance-microscopy (NMR) (Kuchenbrod et al. 1995) and other imaging techniques already used, for example, in phytopathology (Nilsson 1995). An elegant example of the application of imaging of physiological properties in growing leaves is the analysis of the distribution of chlorophyll fluorescence in transgenic plants, in which the expression of the SUT1 gene (sucrose transporter) was reduced by the antisense technique (Kühn et al. 1996). Chlorophyll fluorescence images can be used to obtain maps of photosynthetic activity over entire leaves, including the possibility of analysing its dynamic response analytically (Siebke and Weis 1995). The combination of such techniques with spatio-temporal analysis of growth rates would allow us to study the dynamics and interactions of growth and physiological differentiation much more directly than previously possible.

The kinematic approach has been applied mainly to date in a physiological context, but it is also appropriate for molecular analysis (Rost and Bryant 1996). Here, the situation is even more complicated, because gene expression observed within a certain area of the growth zone might either be related to the actual physiological function within the cells or anticipate functions needed at a later period of the cells' life after they were dislocated by growth. An example which has been studied quite intensively is the expression of genes related to the formation of root hairs in *Arabidopsis* (Dolan and Roberts 1995). In *Arabidopsis* the development of the cellular pattern in the root is quite conservative and has been studied in much detail (Dolan et al. 1993, 1994). In this clearly defined material it has been shown that important genes involved in formation of root hairs are expressed in the meristematic and cell elongation zone of the growing root and not in the root hair zone itself (Galway et al. 1994; Masucci and Schiefelbein 1996). Functional understanding of growth zones is only possible if the spatio-temporal dynamics of these tissues are taken into account.

## 8. Prospects

Spatial and temporal aspects are very closely linked in growing tissues. Therefore, adequate resolution is a prerequisite for the analysis of growth in plant. This is true for the macroscopic distribution of growth rates within a growing zone and for the underlying cellular processes. These determine the orientation of growth and are the functional basis for the morphological differentiation within the growing tissue. In parallel to cellular growth, the physiology of the cells is changed greatly within a very short period of time. Therefore, understanding both physiological and molecular processes in growing zones of plants depends on methods that provide an adequate spatial and temporal resolution of function.

Methods determining spatio-temporal relationships of growth, physiology and gene expression have become available now with modern techniques. The interdependency of cytological, physiological, biochemical and molecular processes is most obvious in growing tissues and therefore fundamental understanding of growth and differentiation in plants requires close interaction of several disciplines.

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## Cytoskeleton: Microtubules

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A decade has passed since the last survey regarding microtubular research in plant cells and tissue was addressed in this series (Kristen 1986). During that time microtubule research gained from two major advancements: a better three-dimensional resolution of the microtubular arrays by confocal laser scanning microscopy and the commencement of the molecular techniques in this area. Both these techniques led to a new challenging move as regards studies on occurrence, arrangement and function of cytoskeletal elements in plant cells. This progress is reflected by an increase in review articles on particular aspects of this research field (Lloyd 1987, 1994; Derksen and Emons 1990; Derksen et al. 1990; Cyr 1991a,b; Hussey PJ et al. 1991; Morejohn 1991; Palevitz 1991; Wada and Murata 1991; Wick 1991a,b; Williamson 1991; Fosket and Morejohn 1992; Lambert 1993; Goddard et al. 1994; Lambert and Lloyd 1994; Cyr and Palevitz 1995). Microtubules can be categorized into four characteristic arrays regarding their arrangement during the life cycle of a plant cell: the array during interphase or in stationary cells, the preprophase band, the mitotic spindle, and the phragmoplast during cytokinesis. In this chapter, essentially questions regarding microtubules in interphase or stationary cells and, where suitable, aspects of the transition between interphase and preprophase will be addressed and those concerning mitosis and cytokinesis only peripherally. With respect to mitosis and cytokinesis readers are referred to recent articles of Lambert et al. (1991), Wick (1991a,b), Samuels et al. (1995) and Staehelin and Hepler (1996). It is not intended to present a complete overview of the related literature published during the last decade. With respect to the topics addressed, the original publications will be quoted or preferably summarizing articles, in case of confirming or extending literature.

### 1. Tubulin

In plants three types of the microtubule forming protein have been identified:  $\alpha$ -,  $\beta$ - and  $\chi$ -tubulin. The initiation of microtubule assembly is promoted by a helically arranged  $\chi$ -tubulin cushion, but microtubules

are basically composed of  $\alpha$ - and  $\beta$ -heterodimers, which form a cylindrical structure by aligning in a polar way head-to-tail and side-to-side, thereby giving rise to a (+) and (-) end. During assembly tubulin monomers are especially added to the (+) end of a growing microtubule.

Coarsely, the  $\alpha,\beta$ -tubulin dimer can functionally be subdivided into two domains, one primarily featuring polymerization aspects and the other associated with regulatory activities. The former includes the intradimer bonding, the lateral association site of the tubulin dimer within the microtubular protofilaments, and the GTP binding site. GTP binding to plant tubulin has yet to be demonstrated although the probable GTP binding sequences seem to be conserved in plant tubulins (Fosket and Morejohn 1992). In animal tissue tubulin polymerization is accompanied by GTP hydrolysis to GDP and  $P_i$ , with the former remaining bound to the  $\beta$ -tubulin whereas a second unhydrolyzed GTP molecule is bound to  $\alpha$ -tubulin (Geahlen and Haley 1979).

The carboxyl-termini of the tubulin dimers and partly the  $\alpha$ -tubulin amino terminus govern most of the centres involved in microtubule dynamics including those for tubulin assembly. Many microtubular properties, however, are mediated by or depend on components which bind to microtubules. The C-termini are highly acidic and therefore excellent domains to associate with cations like calcium or basic polypeptides like the microtubule-associated proteins (MAPs) (Wiche et al. 1991). It is uncertain whether the motor proteins kinesin and dynein also bind to the carboxyl-termini (Vallee and Shpetner 1990). Whereas there is less biochemical diversity comparing plant and animal  $\beta$ -tubulins, the differences regarding biochemical properties between  $\alpha$ -tubulin from brain and from various plants are significant (Little et al. 1984; Picquot et al. 1988; Pal et al. 1990; Fosket and Morejohn 1992).

Early advances in animal and plant microtubule research are closely related to the microtubule poison colchicine which apparently forms a complex with the  $\alpha,\beta$ -tubulin dimer, and it is this tubulin dimer-colchicine complex which destabilizes microtubules (Margolis et al. 1980). In plant cells colchicine only causes the disassembly of microtubules at relatively high concentrations, but herbicides like the phosphoric amide amiprophosmethyl, the dinitroanilines trifluralin and oryzalin, and the N-phenyl carbamates have taken over in plant cells the destabilizing role which colchicine exerts on microtubules in animal cells (Morejohn 1991). All these drugs seem to affect microtubule stability by a similar mechanism, through binding to tubulin (Morejohn 1991). There is little information on the three-dimensional structure of the plant tubulins, although primary sequence data of  $\alpha$ - and  $\beta$ -tubulin were deduced from various plants, including the major research model plant *Arabidopsis*, and the crop plants maize and soybean (Montolieu et al. 1989, 1990; Fosket and Morejohn 1992; Kopczak et al. 1992; Snustad et al. 1992; Villemur et al. 1992).

## 2. Tubulin Gene Families

Cells, tissues and organs undergo specific programmes during differentiation and development which are marked by distinct gene expression. In *Arabidopsis* tubulin gene expression has been shown to be regulated in addition to development orders by environmental changes (Fosket and Morejohn 1992; Goddard et al. 1994). From the three types of tubulin which have yet been characterized in higher plants cells multiple isotypes of  $\alpha$ - and  $\beta$ -tubulin were discovered (Hussey et al. 1991), a situation also found in animal cells (see Sullivan 1988). A detailed comparison of the discovered  $\alpha$ - and  $\beta$ -tubulin genes was presented by Fosket and Morejohn (1992). The amino acid composition of  $\alpha$ - and  $\beta$ -tubulin is homologous to about 35–40% (Little and Seehaus 1988). Both tubulins are acidic proteins with a highly variable C-terminus (Fosket and Morejohn 1992). First indications that tubulin heterogeneity also occurs in plant cells descended from studies to characterize isolated tubulins by gel electrophoresis and subsequent immunoblotting of separated  $\alpha$ - and  $\beta$ -tubulin with monoclonal antibodies (Dawson and Lloyd 1985; Hussey and Gull 1985; Cyr et al. 1987; Hussey et al. 1987; Kerr and Carter 1990). Although isoforms have been reported in several plants they are best studied in *Arabidopsis*, and to some extent in soybean, maize, cotton and pea (Leu et al. 1995). In *Arabidopsis* six  $\alpha$ - and nine  $\beta$ -tubulin genes are expressed (Kopczak et al. 1992; Snustad et al. 1992). Obviously, there is no functional divergence between the observed plant tubulin isotypes, although their amino acid sequence identity is 85–90% for both the  $\alpha$ - and  $\beta$ -isoform when singularly compared. The spatial and temporal expression of the isoforms appears to depend on developmental stages of organs and tissues and on environmental factors like light or temperature (Carpenter et al. 1992, 1993; Joyce et al. 1992). Whereas a  $\beta$ -tubulin gene (Sb1) is up-regulated in soybean hypocotyls (Han et al. 1991), a  $\beta$ -tubulin gene is down-regulated in the same *Arabidopsis* organ via the action of phytochrome (Leu et al. 1995). Low temperature stress causes the down-regulation of the TUB8  $\beta$ -tubulin gene in *Arabidopsis* (Chu et al. 1993). An increase of  $\beta$ -tubulin gene activity was observed in oats after gibberellic acid ( $GA_3$ ) treatment, however, before cell elongation started (Mendu and Silflow 1993). Goddard et al. (1994) suggest that the enhanced expression of tubulin gene families in plants compared with animal cells might be due to the different ability to cope with unfavourable environmental situations such as cold, drought or the light-dark change. Animals are motile and can regulate their body temperature whereas plants are sessile and during evolution only those plants had a chance which learned to adapt to these environmental conditions by, for instance, particular structural changes which involve cytoskeletal elements like microtubules.

Only very recently,  $\chi$ -tubulin genes have been identified in an alga (Vassilev et al. 1995), in ferns (Fuchs et al. 1993; Hoffman et al. 1994) and higher plants (Joshi and Palevitz 1996). The  $\chi$ -tubulins of higher plants show a high sequence homology, about 90%. The molecular mass of the protein is in the range of that of  $\alpha$ - and  $\beta$ -tubulin and amounts to about 53 kDa. In *Arabidopsis* five  $\chi$ -tubulin genes were detected, but only two seem to become expressed (B. Liu et al. 1994). The  $\chi$ -tubulins are thought to play an essential role in microtubule nucleation by forming a template-like basis for the polar assembly of  $\alpha$ - and  $\beta$ -tubulin (Mandelkow et al. 1995; Zheng et al. 1995). Experiments to study the distribution of  $\chi$ -tubulin and thus that of microtubule nucleation sites after antimicrotubular drug treatment support this notion (Liu et al. 1995).

### 3. Microtubule Nucleation

Compared with higher plant cells the spatial control of microtubule array formation seems to be relatively simple in animal and fungal interphase cells in which the (single) centrosome functions as a microtubule organizing centre (MTOC). In plant cells microtubules are not formed at such a template-like organizing centre; therefore, it has been suggested to speak of microtubule nucleation sites (MTNS) to indicate the difference to the situation with respect to the MTOC of animal cells (Lambert and Lloyd 1994). Thus, multiple strategies are conceivable only thinking of the different microtubular patterns during plant cell cycle and the particular rearrangements observed during various cell differentiation processes (Williamson 1991; Cyr and Palevitz 1995). Microtubules are seeded at the nuclear membrane at two distinct phases during the cycle of cells surrounded by a rigid cell wall: at the onset of the interphase, postcytokinesis or M/G<sub>1</sub> transition, and at the end of interphase when competent cells prepare for mitosis during the transition from G<sub>1</sub>- to M-phase (Lloyd 1989; Palevitz 1991; Wick 1991a,b; Lambert 1993; Lambert and Lloyd 1994). In the majority of quiescent cells radial arrays do not occur but are induced in cells around wounded tissue. These cells re-enter the cycle recognizable by the movement of the nucleus from the cell periphery to the cell centre (Lloyd et al. 1991). However, microtubules radiating from the cell cortex to interior regions have also been observed in resting walled cells (Marc et al. 1989; Baluska et al. 1992; Baskin et al. 1992) and they are long known as a feature of non-walled cells like the endosperm cells of *Haemanthus* (Smit et al. 1983). Microtubules of moss protonema cells predominantly reassemble at the nuclear surface during recovery after a previous treatment with microtubule disrupting agencies, but also at the plastidal envelope (Wacker et al. 1988). These cells are, however, characterized by tip growth-like root

hairs and pollen tubes and the nucleus-microtubule association may be stronger due to the different cellular patterning of the microtubules (Derksen and Emons 1990).

These structural indications that the nuclear envelope may function as MTNS are further supported by studies with isolated nuclei which sustain microtubule assembly (Mizuno 1993; Stoppin et al. 1994) and by studies employing antibodies against animal centrosomal proteins which label distinctly microtubule converging sites at the nuclear membrane (Chevrier et al. 1992). Flanders et al. (1990) suggested that microtubules, in general, assemble at the nucleus and are then transported to other cell locations, e.g. during interphase to the cell cortex. This notion is, however, not supported by studies of Marc et al. (1989), Panteris et al. (1991), Hasezawa et al. (1991) and Nagata et al. (1994). Further, fluorescently labelled tubulin microinjected into interphase cells did not reveal tubulin incorporation at the nuclear surface but in the cell cortex and only at the nuclear surface during prophase (Wasteney et al. 1993; Hepler et al. 1993; Hush et al. 1994). Hepler et al. (1993) indicated that part of the cortical microtubules may be generated at cortical sites, which has also been indicated in grass subsidiary cells (Wick 1991b). Additional strong support for MTNS in the cell cortex derived from studies localizing the centrosomal constituent  $\chi$ -tubulin at cortical sites during interphase, as well as in preprophase bands, but at the nuclear membrane apparently only during G<sub>2</sub>-phase (B. Liu et al. 1993, 1994). A similar situation regarding the cortical localization of  $\chi$ -tubulin has been observed in guard cells (McDonald et al. 1993).

From these studies it can be concluded that the nuclear surface functions as MTNS during plant cell cycle at defined periods, but microtubules are also nucleated at the cell cortex. All corresponding notions, however, need further experimental verification, mainly the questions: (1) are microtubules which were previously assembled at the nuclear surface translocated to and anchored at the cell cortex? And/or (2) is centrosomal material, for instance  $\chi$ -tubulin, moved to the cortex in association with translocated microtubules giving rise to the observed MTNS in the cortex? In contrast, Smirnova and Bajer (1994) proposed a different model: centrosomal material, including  $\chi$ -tubulin, is not moved in plant cells, but according to their model assembling plant microtubules associate with their minus ends to tree-like converging structures which then self-arrange into expanded arrays. Y-shaped centres frequently observed in the cell cortex during recovery from microtubule disrupting agencies assist this notion (Falconer et al. 1988; Williamson 1991; Smirnova and Bajer 1994).

All the evidences regarding the occurrence of MTNS in higher plant cells are obtained more or less by indirect measures. A clear picture will only be gained if MTNS can be visualized more directly, for instance, by making use of biochemically fluorescently labelled  $\chi$ -tubulin synthe-

sized, e.g. by molecular biological means. The question of MTNS-scattering from the nuclear membrane to other cellular locations will only be resolvable by procedures which will allow us to follow MTNS distribution throughout the cell cycle of meristematic cells and possibly during differentiation processes of cells designed for a particular function in the organism.

#### **4. Microtubule Associated Proteins (MAPS)**

Microtubules are certainly involved in many developmental and cellular events. They are constructed in the form of a stiff, tube-like structure which seems to bear little dynamic properties. The majority of the so-called microtubular functions are, however, due to the capability of the microtubules to associate with numerous other proteins, the MAPs (see below; Cyr 1994; Cyr and Palevitz 1995). There are only a few incidents propelled by the turnover of the microtubular structure, e.g. the break-down into subunits and their reassembling into new microtubules with, for instance, a new orientation. MAPs have obviously been subjected to manifold genetic variations whereas the tubulin-composed backbone has rather been preserved during evolution, even between kingdoms (Wiche et al. 1991; Fosket and Morejohn 1992; Raff 1994; Mandelkow and Mandelkow 1995).

##### **a) Classification**

A mutual understanding is required of which proteins should be classified as MAPs due to the high diversity of these proteins and to the acidic nature of the tubulins which may favour unspecific association of basic polypeptides with microtubules. Apparently, there is some need to lay down criteria for their identification and characterization (Olmsted 1986; Cyr 1991b; Cleveland 1993). In the past, MAPs have been defined in a relatively narrow (Morejohn 1994) or broad sense (Olmsted 1986; Cyr 1991b; Durso and Cyr 1994c). Morejohn (1994) refers to the definition of Cleveland (1993) that only those proteins should be named MAPs which have been proven by immunological means to co-localize intracellularly with microtubules. However, even this criterion alone may still be inconclusive because the association could be a temporary property of microtubules and could, therefore, easily be missed.

For the moment, it is suggested we refer to 'putative MAP' as long as any classification is premature with respect to appropriate biochemical, immunocytochemical and genetic measures for any conceivable plant MAP. Eligible assays might include (1) binding to microtubules in situ and in vitro, for example in the presence of a non-hydrolyzable analogue

of ATP, (2) the search for the occurrence of tubulin-binding motifs (see below), and (3) the attribution of a function in microtubule performance. As more criteria support the classification, the more reliable will be the MAP grouping of any protein in question.

#### b) Binding and Bundling Proteins

Several higher plant proteins bind *in vitro* to taxol-stabilized brain microtubules (Cyr and Palevitz 1989; Cyr 1991a; Yasuhara et al. 1992; Durso and Cyr 1994a; Nick et al. 1995) as well as to plant-derived microtubules (Vantard et al. 1991; Schellenbaum et al. 1992, 1993; Hugdahl et al. 1993; Jiang and Sonobe 1993; Durso and Cyr 1994b). The 65-kDa protein of tobacco BY-2 cells studied by Jiang and Sonobe (1993), the 100- and 50-kDa proteins from maize (Nick et al. 1995) and the protein translocation factor EF-1 $\alpha$  (Durso et al. 1996) have been shown to meet both aspects to bind to isolated and to co-localize intracellularly with microtubules. All four microtubule arrays became decorated by a 120-kDa MAP isolated from carrot suspension cells (Chan et al. 1996). The molecular weight of putative MAPs identified in maize suspension cells range from 39 to 125 kDa (Schellenbaum et al. 1993). Immunoblot studies indicated that a 83-kDa putative MAP is related to the neural MAP tau (Vantard et al. 1991). A putative 100-kDa MAP from maize cells has been reported to be heat stable (Vantard et al. 1994), but this property can apparently not be generalized regarding plant MAPs because Nick et al. (1995) demonstrated that the 50-kDa MAP from the same cell line is heat sensitive but meets other MAP features.

As pointed out by Lambert and Lloyd (1994) the ability of plant MAP to coassemble with neural or plant tubulin may indicate that the tubulin-binding domains are possibly conserved in MAPs through evolution. Fosket and Morejohn (1992) also suggested a similarity of the microtubule-binding motifs because of the highly conserved amino acid sequence of tubulin. Plant microtubules are characterized by more or less strong bundling properties during particular situations, e.g. the preprophase band in meristematic cells and distinguished microtubule arrays during vessel cell differentiation (Williamson 1991). Since many of the putative plant MAPs reported so far are described to bundle microtubules *in vitro*, this is possibly a major function *in vivo*. Animal MAPs can cause tubulin polymerization and stabilization in unfavourable conditions, for instance in the presence of low tubulin concentration (Olmsted 1986; Fosket and Morejohn 1992; Cleveland 1993; Cyr 1991b; Hugdahl et al. 1993). Chan et al. (1996) have recently shown that a MAP fraction isolated from carrot suspension cells stimulated tubulin polymerization at concentrations lower than otherwise necessary for self-assembly *in vitro*. The stabilization function has not been satisfactorily



addressed to any of the putative plant MAPs. However, MAPs were reported to be involved in microtubule destabilization in detergent-lysed carrot protoplasts and have been indicated to be calcium/calmodulin dependent (Cyr 1991a). A calcium/calmodulin-regulated MAP, named STOP (stable tubule only polypeptide) is known from neural cells (Pirollet et al. 1992). Regarding the cold stability of microtubules in plant cells, a similar mechanism seems conceivable.

### c) Motor Proteins

In animal cells vesicles are transported along microtubules due to the activity of two well characterized motor proteins, dynein and kinesin; both resemble features of a classical MAP (Holzbaur et al. 1994; Pereira and Goldstein 1994; Scholey and Vale 1994). The situation regarding the occurrence of either of these two MAPs in higher plant cells is still in its infancy. Evidence for kinesin-related proteins has been obtained from studies with tobacco BY-2 cells (Asada et al. 1991) and tobacco pollen tubes (Tiezzi et al. 1992; Cai et al. 1993; G. Q. Liu et al. 1994). A microtubule translocating activity has, furthermore, been isolated from phragmoplasts of tobacco cells (Asada and Shibaoka 1994). The kinesin-related protein from tobacco pollen tubes seems to possess microtubule activated ATPase activity. This 100-kDa protein is recognized by a monoclonal antibody raised against the kinesin heavy chain from bovine brain (Cai et al. 1993; G.Q. Liu et al. 1994). The occurrence of a kinesin-related gene family has been demonstrated in *Arabidopsis* and tobacco BY-2 cells (Mitsui et al. 1994, 1996; Liu et al. 1996). The gene products showed high sequence homology to the motor domain of the animal kinesin heavy chain and to features of helical-coiled-coils of filamentous polypeptides (Pereira and Goldstein 1994; Scholey and Vale 1994). A recently cloned plant motor protein from *Arabidopsis*, *Solanum* and tobacco named "kinesin-like, calmodulin-binding protein" (KCBP) displays a distinct, yet not observed, form of regulation (Reddy et al. 1996a,b; Wang et al. 1996). The conserved motor domain resides in the carboxyl terminus with an adjacent calmodulin-binding domain. As other kinesins with a C-terminal motor domain, this kinesin-like protein should be directed towards the minus end of microtubules.

With respect to dynein, yet, it could only be demonstrated that plant microtubules can be translocated by animal-derived dynein (Yokota et al. 1995). Two high molecular weight polypeptides of tobacco pollen tubes are noticed by an antibody which recognizes a conserved sequence in the ATP-binding site of dynein heavy chain (Moscatelli et al. 1995). The evidence for MAP-regulated microtubule dynamics in higher plant cells are increasing and by employing biochemical, cytological and mo-

lecular procedures our understanding will hopefully improve in the near future.

## 5. Organization of Cortical Microtubules

Besides the spindle apparatus the cortical microtubule arrays observed in different types of higher plant cells have always intrigued researchers. This microtubular pattern not only occurs during interphase of meristematic cells but also particular modifications of its basic arrangement seem to be indispensable for various morphogenetic events regarding organ development or cell differentiation (Williamson 1991; Cyr 1994; Goddard et al. 1994; Lloyd 1994; Cyr and Palevitz 1995). Aspects of cortical microtubule organization involve (1) their origination and site of assembly, (2) their linkage to the plasma membrane, (3) their lateral density and the association among each other and to other cellular components, and, of course, (4) their functional role. Ever since the first microtubules have been described in plant cells, cortical microtubules have been considered to be, by some means, involved in cellulose deposition (Ledbetter and Porter 1963; Robinson and Quader 1982), although this concept has been disputed in a more general sense (Roland and Vian 1979) or only with respect to tip growing systems (Derksen and Emons 1990). The deposition of the rigid cell wall mainly achieved by the threading of the crystalline cellulosic microfibrils into the cell wall matrix efficiently directs and supports plant morphogenesis (Green and Selker 1991). Cell expansion and thus cell shape depends on a concerted action between turgor pressure and the controlled extracellular disposition of cellulose microfibrils. Besides guiding the deposition of cellulose the cortical microtubules may also contribute to strengthening the cell cortex before the cell wall has been completed.

Cortical microtubules have to fulfil several assumptions in order to accomplish these presumed functions. They must force and maintain an appropriate organization of cellular constituents in the cortex in close proximity to the plasma membrane but still retain enough flexibility to be able to respond to external or internal constraints. Therefore, they are in close contact with the plasma membrane, to some extent laterally with each other, and rearward into the cytosol to anchor cortical events to the rest of the cytoplasm. These interactions do not have to be of permanent nature because dynamic properties are required to respond to developmental signals, e.g. growth regulating substances, light, temperature, gravity, nutritional shortages or even a pathogenic attack.

Two situations of cortical microtubule rearrangement are known: (1) the formation of significant bundles, and (2) the change of their orientation with respect to the major axis during cell elongation growth. Questions regarding the molecular and biochemical basis are only beginning

to become untied (see Shibaoka 1991; Williamson 1991; Cyr 1994; Cyr and Palevitz 1995). How does the cell achieve the reorientation of the cortical microtubules? What are the regulatory steps and which are the cytosolic components involved to force and maintain the new orientation?

#### a) Reorientation and Bundling: The Phenomenon

Cell growth is mainly affected and regulated by hormones such as auxin, gibberellic acid, ethylene, abscisic acid, jasmonic acid and brassinolides. They either induce, support or stop axial expansion growth and possibly set a path to alter growth direction (Quatrano 1987). Microtubules are thought to receive and pass on signals to assist the process of cell wall patterning and thus cell morphology (Williamson 1991; Lloyd 1994). During the past, many aspects regarding changes of microtubule orientation in combination with elongation growth have been reported but also after applying physical stress (Hush and Overall 1991). The effect of gibberellic acid ( $GA_3$ ), which induces longitudinal cell expansion, on microtubule orientation has been studied in epicotyl cells of azuki bean and pea (Mita and Shibaoka 1993), maize mesocotyls (Mita and Shibaoka 1984; Ishida and Katsumi 1991), cucumber hypocotyls (Ishida and Katsumi 1992) and oat coleoptiles (Iwata and Hogetsu 1989). In all these systems the hormone causes the cortical microtubules to shift from a longitudinal to a transverse pattern with respect to the cell axis.

Microtubule bundling is observed at the sites of secondary wall modification by augmented cellulose deposition, for example during the differentiation of vessel cells, for instance during conifer tracheid differentiation (Abe et al. 1995), or during the period of preprophase band happening, the  $G_2/M$ -phase transition (Giddings and Staehelin 1991; Williamson 1991; Cyr 1994). The latter has also been discussed in relation to cell wall changes because at the site of the preprophase band the new cell wall will merge with the parental wall. The preprophase microtubule bundle is thought to function as a temporary support for that region and may direct the lay down of additional supporting cell wall material (Mineyuki and Gunning 1990; Sawidis et al. 1991). The mechanical and functional attributions of this alignment need to be resolved in order to understand the complexity of its postulated role. Microtubule bundling MAPs probably achieve the lateral connection between single microtubules to maintain the dense side-by-side arrangement (see Sect. 5), but the involved cohesion forces are unknown. A similar situation may exist regarding the lateral microtubule aggregation adjacent to sites reinforced by newly deposited wall material, mainly cellulose fibrils (Giddings and Staehelin 1991). This has been best studied in *Zinnia elegans* cultures which can be induced to differentiate into

xylem elements (Falconer and Seagull 1988; Fukuda and Kobayashi 1989).

#### b) Reorientation and Bundling: A Common Mechanism?

The mechanisms leading to denser grouping of microtubules are still poorly understood. Reorientation of microtubules may involve similar activities. Both events can be explained to occur either by (1) break down of existing microtubules to tubulin (dimers?) and their subsequent reassembly into a more densely packed or differently oriented array, or (2) moving the microtubules on the whole or as fragments to their new site of destination.

In this respect, the length of the cortical microtubules is an important feature considering its constitution or any transition forced by physiological conditions later on. The estimated values for most plant cell microtubules are in the range of 1–10  $\mu\text{m}$  (see Williamson 1991), although extremely long cortical microtubules have been observed in seed hairs of *Cobea* (Quader et al. 1986). The visualization of the cortical arrays by the immunofluorescence technique only gives an impression of the overall alignment, but details of the alignment of single microtubules in this array, the number or the length of single microtubules cannot, of course, be estimated from such images. Microtubular fragments were reported to occur during the process of aggregation (Seagull et al. 1987). Unfortunately, the techniques used until recently did not furnish a clear picture of these dynamic processes. Nevertheless, movement of fragments or whole microtubules may result from sliding of microtubules along each other, thus pushing each other closer together or into a new orientation. So far, with respect to plant cells a nucleotide-driven microtubule translocating activity, observable in studies employing animal-derived microtubules, has, hitherto, only been isolated from phragmoplasts of tobacco BY-2 cells (Asada and Shibaoka 1994).

#### c) A Role for Actin in Microtubule Patterning?

A role for actin filaments in organizing or/and maintaining the cortical microtubule array has lately been discussed even in view of the fact that distinct links exist between cortical microtubules and the plasma membrane (Williamson 1991; Cyr 1994). Dot-like actin spots have been observed in differentiating xylem tracheary cells forming a net-like pattern with microtubules which apparently changed their orientation (Fukuda and Kobayashi 1989; Derksen et al. 1990). This notion is further supported by experiments employing the actin filament poison cytochalasin B which affects the microtubule pattern in differentiating cells (Seagull

1990; Wernicke and Jung 1992). Actin filaments have themselves been demonstrated in the preprophase bands of meristematic onion root cells (Mineyuki and Palevitz 1990; Ding et al. 1991; Eleftheriou and Palevitz 1992). In the presence of the actin filament poison cytochalasin D the lateral extension of the preprophase band expands, indicating a close engagement of both cytoskeletal elements in the cell cortex during this phase of the life cycle. Actin also seems to be associated with plant plasma membrane as has biochemically been deduced from isolated plasma membrane vesicles (Abe et al. 1992; Sonesson and Widell 1993).

#### d) Speediness of Microtubule Turnover and Reorganization?

Microtubule break down and reassembly infers the existence of MTNS at the plasma membrane. In a broad sense, a break-through in this respect took off with the work of Zhang et al. (1990) who studied the incorporation and turn over of fluorescently labelled brain tubulin microinjected into living *Tradescantia* stamen hairs. Brain tubulin not only co-assembled with genuine plant tubulin into a particular set of microtubules but also co-polymerized into spindle, phragmoplast and cortical microtubules (Zhang et al. 1990; Wasteney et al. 1993). Photobleaching studies with thus labelled microtubules showed that the cortical microtubules of plant cells turned over two to three times as rapidly as animal interphase microtubules (Hush et al. 1994). This finding agrees well with the observation that the cortical microtubules of pea epidermal cells become equally labelled within about 20 min after rhodamine-conjugated brain tubulin has been microinjected (Yuan et al. 1994). The result of incorporation and photobleaching experiments correspond nicely with the discovery of putative MTNS at the plasma membrane by the detection of  $\chi$ -tubulin spots indicating that nucleation sites and the resulting cortical microtubules are located in the same cellular region (Liu et al. 1993).

Microinjection studies in combination with confocal sectioning of cells, for the first time, allowed to follow directly the change of microtubule orientation from longitudinal to transverse after inducing cell elongation by gibberellic acid. Previously, the chronology of orientation shifts caused by hormones or extracellular constraints could only be studied by keeping the fixation intervals of successive probes as short as possible. This way transition times less than 10 min have been observed for treatments with ethylene (Roberts et al. 1985), or auxin, and different light regimes (Zandomeni and Schopfer 1993). The microtubules do not shift to their new orientation in a single step but move through a stage of discordant alignment (Lloyd et al. 1996). During this transition, the newly oriented microtubules must be protected for disassembly, whereas the old ones may lose previous stability shields and may then gradually

disappear. As already observed earlier by Bergfeld et al. (1988), cortical microtubules facing the outer epidermal cell wall may change to an alignment with a steeper angle to the cell axis than those of the radial walls. The angles, however, approximate when they join at the corners (Yuan et al. 1995).

#### e) Regulation of Microtubule Stability

Two mechanisms are known from animal cells which lead to differential stability of microtubules: modification of the tubulins and the association with particular MAPs. The identification and characterization of the latter is still in its infancy regarding plant cells (see Sect. 4) and, thus, there is only little biochemical information on plant cell MAPs with stabilizing properties. Axonemal microtubules are possibly the most stable ones. This property was attributed earlier to the covalent modification of the  $\beta$ -tubulin which is acetylated on lysine-40. However, acetylation is seen more in the light of  $\beta$ -tubulin preconditioning for the association with specific MAPs which cause microtubule stabilization. The latter may also apply for the well established modification of the carboxyterminus of the  $\alpha$ -tubulin, the tyrosination/detyrosination cycle (Thompson 1982) where in vitro studies showed that detyrosination alone did not stabilize microtubules (Webster et al. 1990). After gibberellic acid treatment  $\alpha$ -tubulin isotypes could not be detected anymore in a pea mutant (Duckett and Lloyd 1994) and in azuki bean hypocotyl cells (Mizuno 1994) by an antibody which recognizes the tyrosinated carboxyterminus of the  $\alpha$ -tubulin. Thus, gibberellic acid treatment causes the detyrosination of  $\alpha$ -tubulin isotypes which may be responsible for the stabilization of the newly oriented microtubules during gibberellic acid-induced elongation growth. This modification may, however, only qualify the tubulin isotypes for the association with particular MAPs. In animal cells three types of MAPs have been characterized with respect to stabilization properties: the STOP proteins (stable tubule only protein), the myelin basic protein (MBP) (Pirollet et al. 1992) and the histone H1 which has been found in axonemes (Multigner et al. 1992). The search for the occurrence of such proteins in plant cells will be an exciting challenge for the future.

Further, phosphorylation/dephosphorylation activities may be involved in the regulation of microtubule stability. Protein kinases, for instance the  $p34^{cdc2}$  kinase, have been demonstrated to occur in the spindle but also in the preprophase band (Mineyuki et al. 1991; Colasanti et al. 1993). If phosphorylation/dephosphorylation plays a role in cortical microtubule function, then one would rather suspect the involvement of mitogen-activated protein kinase cascades (Nishihama et al. 1995).

## 6. Concluding Remarks

During the past, many phenomenological aspects of developmental and physiological incidents involving microtubules have been described, although many questions awaited still an answer. These days, plant microtubule research apparently is in an exciting period because now the tools seem to be at hand to explore questions concerning the regulation of microtubule structure, arrangement and function in more detail with the aid of the molecular techniques and the microinjection method in combination with confocal microscopy. Through the latter, first hints regarding the dynamics of cortical array orientation, microtubule associated proteins, the sites of microtubule nucleation and some fundamental aspects of plant tubulin biochemistry have been obtained.

Future progress will likely concern plasma membrane-microtubule linking proteins, the occurrence of MAPs and their function, the setting of nucleation sites and the interaction with other cytoskeletal elements in the cell cortex or elsewhere in the cell. What defines a microtubule nucleation site, and, in the case of  $\chi$ -tubulin involvement, how is this tubulin moved to and recognized at its destination site? Are there any rules with respect to the positioning of microtubules within an array? Probably MAPs with motor and structural functions such as the bundling property may have a major role in this and related processes. The very recently indicated  $\text{Ca}^{2+}$ /calmodulin regulation of, for instance, kinesin may turn out to be a more general regulatory feature of microtubule function, possibly including phosphorylation/dephosphorylation cycles; the latter especially in view of the transduction of developmental or growth signals which involve microtubule actions.

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# The Physiology of Tropisms\*

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## 1. Introduction

The historic work of Darwin, a century ago, has set a landmark in the field of plant movements, particularly in the category of directed growth (Darwin 1896). Plants and fungi through directed growth, defined as "tropism", respond in spatial orientation to environmental stimuli such as light, gravity, temperature and water (Poff et al. 1994). The field has been reviewed by Hensel (1986) for *Progress in Botany* in vol. 48; for reviews in fungi, lower and higher plants see also Konings (1995); Fukaki et al. (1996); Estelle (1996); Sievers et al. (1996).

The different stimuli show up in the nomenclature as photo- gravi-, thermo- and hydrotropism (Haupt 1996). The tropism by the plant is an integrated response to a number of pieces of information concerning the status of the plant's environment. Through signal integration, physically different stimuli – such as light simultaneously with gravity – may be involved along with epinasty (Haupt 1996) in orienting, e.g., the leaves such that the upper surface of the leaf faces the light source. Tropisms have been widely studied, largely because they are obvious outward evidence of the processing of information by the plant.

## 2. Phototropism

The phototropic response is an important component of seedling establishment in higher plants because it orients the young seedlings for maximal photosynthetic light capture. In the life cycle of fungi and lower plants, direction of spore release and germling spread often is determined by phototropic bending.

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\* Dedicated to Prof. Pill-Soon Song on the occasion of his 60th birthday.



### a) Identity of the Photoreceptor Pigment

Action spectra for phototropism in *Avena* (Thimann and Curry 1961) and in the fungus *Phycomyces* (Curry and Gruen 1959; Delbrück and Shropshire 1960) are quite similar. In general, the spectra show peaks in the blue region, a peak at 370 nm, little action at wavelengths higher than 500 nm, and a peak in the UV range at 280 nm (Dennison 1979). On the basis of such spectra, the responsible pigment has been referred to as the blue-light photoreceptor pigment (see, e.g., Schmidt 1984). The consensus in the literature has been that these spectra most closely match the absorption spectrum of a flavoprotein in a relatively hydrophobic environment (Dennison 1979), but pterins as the chromophoric group (and carotenoids) are a matter of discussion. The 280-nm band is thought to be a consequence of absorption by the protein, and the 370-nm band and those in the blue are thought to result from absorption by the flavin or pterin or carotenoid moiety (Dennison 1979; Galland and Senger 1988; Quiñones and Zeiger 1994).

Many efforts have been directed toward the identification of the blue-light photoreceptor pigment involved in phototropism (for review, see Pohl and Russo 1984). Flavins and pterins have some of the attributes expected for a photoreceptor mediating blue-light-induced phototropism in plants (Galland and Senger 1988). Besides the classical photoreceptor candidate,  $\beta$ -carotene, corn coleoptiles contain many other carotenoids, including the main components of the xanthophyll cycle, violaxanthin and zeaxanthin (Quiñones and Zeiger 1994). Here, dark-grown coleoptiles accumulated violaxanthin, but lacked zeaxanthin. Coleoptiles devoid of zeaxanthin did not bend in response to a blue-light pulse. Coleoptile tips converted violaxanthin into zeaxanthin in the light. Manipulation of coleoptile zeaxanthin content by red light, red light plus darkness, or incubation with the inhibitor of zeaxanthin formation, dithiothreitol, resulted in a blue-light-induced bending that was proportional to zeaxanthin content. However, Palmer et al. (1996) gave evidence that zeaxanthin is not the photoreceptor for phototropism in maize coleoptiles.

The conclusion is that the blue-light receptor of phototropism to a great extent remains unknown to date (Galland 1992). This situation gave rise to efforts to identify mutants first in *Phycomyces* and *Arabidopsis thaliana* with alterations in the photoreceptor pigment for phototropism. Based on their comparative analysis of action spectra for the wild type and several mutants, Galland and Lipson (1985a,b; 1987) reached the conclusion that multiple photoreceptor pigments are involved in *Phycomyces* phototropism. The same conclusion has been reached for *Arabidopsis thaliana*, based on an analysis of the wavelength dependence for the fine structure of the fluence response relationship (Konjević et al. 1989). The *Arabidopsis thaliana* mutant, JK224, exhibits

an apparent alteration in one photoreceptor pigment, while a second pigment in this mutant appeared unchanged (Konjević et al. 1992). Recognition that phototropism is mediated by multiple photoreceptor pigments should greatly assist toward real progress in the identification of these pigments.

Liscum and Briggs (1995, 1996) reported on the isolation of eight mutants of *Arabidopsis* that lack or have severely impaired phototropic responses. These *nph* (nonphototropic hypocotyl) mutants comprise four genetic loci: *nph1*, *nph2*, *nph3* and *nph4*. Physiological and biochemical characterization of the *nph1* allele series indicated that the *NPH1* locus may encode the apoprotein for a dual- or multi-chromophoric holoprotein photoreceptor capable of absorbing UV-A, blue and green light, and that this photoreceptor regulates phototropic response of *Arabidopsis*. It appears that the *NPH1* protein is a 120-kDa phosphoprotein (see below and Sect. 2c) because all of the *nph1* mutations negatively affected the abundance of this protein. In addition, the putative *NPH1* photoreceptor protein is genetically and biochemically distinct from the *HY4* protein (Ahmad and Cashmore 1993), which most likely acts as a photoreceptor for blue-light-mediated hypocotyl growth inhibition. Furthermore, the *NPH1* and *HY4* proteins are not functionally redundant because mutations in either gene alone affect only one physiological response but not the other, thus providing strong support for the hypothesis that more than one blue-light photoreceptor is required for the normal growth and development of a seedling. In *Phycomyces blakesleeanus*, Campuzano et al. (1996) inferred that an intact near-UV/blue-light photoreceptor system is required even in darkness for negative gravitropism, the ethylene response and autochemotropism.

Fine analysis of the photochemical reaction cycle of the phototropic sensory pigment was started by Galland et al. (1995). Sporangiophores of *Phycomyces blakesleeanus* are sensitive to near UV and blue light. The quantum effectiveness of yellow and red light is more than six orders of magnitude below that of near UV or blue light. Phototropism mutants with a defect in the gene *madC* are about  $10^6$  times less sensitive to blue light than the wild type. These mutants respond, however, to yellow and red light when the long wavelength light is given simultaneously with actinic blue light. In the presence of yellow or red light the photogravitropic threshold of *madC* mutants is lowered about 100-fold, though the yellow and the red light alone are phototropically ineffective. A step-up of the fluence rate of broad-band red light ( $> 600$  nm) from  $6 \times \text{mW} \cdot \text{m}^{-2}$  to  $6 \text{ W} \cdot \text{m}^{-2}$  elicits, in mutant C148 *madC*, a transient deceleration of the growth rate. The growth rate of the wild type is not affected by the same treatment. The results were interpreted by Galland et al. (1995) in terms of a red light absorbing intermediate of the blue-light photoreceptor of *Phycomyces*. The intermediate should be short-lived in the wild type and should accumulate in *madC* mutants.

Well compatible with the concept of multiple blue-light photoreceptor pigments are the findings of Martinrojas et al. (1995): Calculations of absorbed-energy distributions in the sporangiophore of *Phycomyces* showed that this zygomycete fungus responds differently to similar spatial distributions of blue and UV radiations. Wild-type and mutant sporangiophores had the same high UV absorption due to gallic acid. The authors conclude that UV tropism is not just a modification of blue phototropism due to the high UV absorption of the sporangiophores. *Phycomyces* has a separate sensory system responsive to UV radiation, but not to blue light. Vegetative spores of *Phycomyces* were used by Galand (1996) to investigate UV-killing and photoreactivation. Irradiation with far-UV light generated shouldered exponential survival curves. Post-irradiation with near-UV, blue or white light caused photoreactivation, i.e. increase of the survival rate. Photon fluence-response curves for photoreactivation, which were generated for selected wavelengths between 350 and 610 nm of light, were generally monophasic, while they were biphasic when 385-nm light was used. A seven-point action spectrum for photoreactivation displayed a maximum at 385 nm indicating that the DNA-photolyase of *Phycomyces* contains as chromophores 5,10-methenyltetrahydrofolate (MTHF) and FADH-2. Interestingly, two light-insensitive mutants, C47 *madA* and C111 *madB*, which were defective for phototropism and photodifferentiation, also lacked photolyase-mediated photoreactivation.

Recently, the laboratories of Briggs and Hager, respectively, made what appears to be a major progress toward identification of one of the photoreceptor pigments (see also Sect. 2.c). A 100-kDa protein (Hager and Birch 1993) and/or 120-kDa protein (Palmer et al. 1993a,b), associated with the plasma membrane, is phosphorylated during exposure to blue light. Although first observed in pea (Warpeha et al. 1992), phosphorylation of the protein is also seen in a number of other plants, including *Arabidopsis thaliana* (Reymond et al. 1992). Phosphorylation is found at approximately wild-type levels in JK218, an *Arabidopsis thaliana* mutant that exhibits no phototropism; this demonstrates that phosphorylation is not a consequence of phototropism. Phosphorylation at approximately 5% of the wild-type level, however, is seen in JK224 which is thought to be a single photoreceptor pigment mutant (see above). Thus, the blue-light-induced phosphorylation of a 100–120-kDa protein appears to be closely connected to one specific blue-light photoreceptor pigment that mediates phototropism in *Arabidopsis thaliana*. The protein behaves as a kinase that phosphorylates itself (Hager and Birch 1993; Palmer et al. 1993a,b). The blue-light-absorbing chromophore appears to be specifically associated with the kinase in solution, but it is not yet certain that the chromophore migrates with the kinase in Sodium Dodecyl Sulphate (SDS) gel.

The next obvious step is to clone the gene for the 100–120-kDa protein. If transforming the mutant JK224 with the wild-type gene restores wild-type levels of blue-light-induced phosphorylation and wild-type phototropism, it can then be concluded that the 100–120-kDa protein is required for phototropism, and the chromoprotein associated with this autophosphorylatable protein is likely to be one of the phototropic photoreceptor pigments. The remaining challenge will then be to identify the other phototropism photoreceptor pigment(s).

The recent identification of a gene coding for a blue-light photoreceptor pigment involved in the blue-light suppression of hypocotyl elongation in *Arabidopsis thaliana* (Ahmad and Cashmore 1993) is of considerable interest. Although the photoreceptor pigments for phototropism appear to be distinct from the hypocotyl suppression photoreceptor pigments (Liscum et al. 1992), they may belong to the same family of related pigments. Thus, we may finally be approaching the elusive light photoreceptor pigments in phototropism (Briggs and Liscum 1997).

#### b) Zone of Light Sensitivity

From the point of photoreceptor evolution and blue-light-mediated signal transduction, analysis of cryptogamic plants appears highly desirable. Positive phototropism in the thallus of the marine coenocytic green alga *Bryopsis plumosa* was investigated by Wada and co-workers in terms of the mode of bending, the photosensitive zone and the effectiveness spectrum (Iseki et al. 1995a). The bending occurred as a consequence of a difference in growth rate between the illuminated and the shaded sides of the thallus. Elongation on the shaded side was stimulated while that on the illuminated side was inhibited. However, the overall elongation rate was barely affected. Illumination with a microbeam revealed that a zone from approximately 80–120  $\mu\text{m}$  below the tip was the most photosensitive one. The effectiveness spectrum showed that blue light ( $< 550 \text{ nm}$ ) was most effective, with light at 467 nm having maximal effectiveness.

The same laboratory analysed the negative phototropism in the rhizoid of *Bryopsis plumosa* in the range of visible to blue light (Iseki et al. 1995b). The growth zone of the rhizoid was confined to the apical hemisphere, as is typical of tip growth. Upon unilateral illumination, the rhizoid bent away from the light source with a "bulging" manner. The photoreceptive site for phototropism was also restricted to the apical hemisphere. The action spectrum for this negative phototropism was determined from fluence-response curves that were obtained after fixing the duration of illumination at 60 min and varying the fluence rate between 0.1 and 3.0  $\text{W m}^{-2}$ . The action spectrum had a large peak at 467 nm and

smaller peaks at 378 and 414 nm, resembling the action spectra of "typical" blue-light responses.

The rhizoid of *Bryopsis plumosa* exhibited negative bending that was due to bulging upon absorption of light in the UV region, as well as in the visible region, of the spectrum (Iseki and Wada 1995). The negative bending might not be a result of the inhibition of growth on the irradiated side of the apical hemisphere by UV irradiation because growth inhibition was observed after bending had reached a maximum within 1–2 h. The action spectrum obtained from fluence rate-response curves had a pronounced peak at 260 nm and a small peak at 310 nm. The quantum effectiveness at 260 nm was about five times that in the visible region. Phenylacetic acid (PAA), a potent inhibitor of flavin photoreaction, inhibited the phototropic response to both UV light and blue light without any obvious effect on tip growth. The inhibition of the phototropic response to blue light by PAA was partially overcome by rinsing the alga with riboflavin-containing medium which suggests the involvement of flavins in the phototropism of *Bryopsis* rhizoids.

Phototropism of young *Adiantum* fern leaves was induced by red light as well as by blue light. The blue light response was mainly mediated by the "typical" blue-light-absorbing pigment, while the red light response was mediated by phytochrome. This is the first evidence of phytochrome action in diploid fern tissue (Wada and Sei 1994).

### c) Elements of the Transduction Chain

Not one element in the phototropism transduction chain is known with certainty. Much of the effort in this arena has centred on the Cholodny-Went theory, which is based on the independent work of Cholodny (1927) and Went (1928). In this theory, tropistic curvature is a consequence of the movement of a growth substance (auxin) from one side of the organ to the other side. An increase in auxin concentration leads to an increased cellular elongation, whereas a decrease in auxin concentration leads to a decreased cellular elongation. This differential in cellular elongation on the two sides of the organ results in curvature of the organ. In the case of phototropism, the auxin is proposed to move from the lighted side to the shaded side. This results in increased cellular elongation on the shaded side, decreased cellular elongation on the lighted side and, thus, curvature towards the source of light. In photo- and gravitropism, the unequal distribution is proposed to result from a transverse polarization of the cells, which results in lateral transport of auxin (Went and Thimann 1937). Hasegawa and co-workers studied the structure-activity relationships of the naturally occurring auxin-inhibiting substance, 6-methoxy-2-benzoxazolinone, isolated from maize shoots, and its artificial analogues with respect to auxin activity

and membrane-bound auxin-binding protein (Hoshisakoda et al. 1994). Competition by benzoxazolinones with an alkoxy group at C-6 with H-3-NAA at auxin-binding protein(s) isolated from endoplasmic reticulum (ER) membrane of maize shoots showed a positive correlation with their physiological effects. However, since the inhibitory activity of the benzoxazolinones for auxin-receptor(s) binding was small compared with their physiological activity, the benzoxazolinones may contribute to inhibition of auxin-induced growth through interference with other auxin-receptors, not identified to date (Hoshisakoda et al. 1994).

A number of papers have argued recently against the Cholodny-Went theory. These arguments have been based on evidence or proposals that growth regulators other than auxin are involved in tropisms (Bruinsma et al. 1975; Hasegawa and Togo 1989), that the concentration of auxin on the lighted side of the shoot is the same as that on the shaded side (Togo and Hasegawa 1991; Hasegawa and Yamada 1992), and/or that the difference in auxin concentration is insufficient to account for the observed changes in growth rate on the two sides. Therefore, considerable controversy continues to surround the Cholodny-Went theory. This controversy has been best summarized recently in a multi-author forum (Trewavas et al. 1992).

Alternatives to the Cholodny-Went theory are the theory of Boysen Jensen (1928) and the theory of Blaauw (1918) and Paál (1919). The Boysen Jensen theory predicts an increased rate of cellular elongation on the shaded side of the organ in phototropism with no change on the lighted side (Boysen Jensen 1928). The Blaauw-Paál theory predicts a general decrease in cellular elongation with a greater decrease on the lighted side than on the shaded side (Blaauw 1918; Paál 1919). Thus, as has been discussed by Pohl and Russo (1984), these theories should be easily distinguished by careful measurements of growth rate on the two sides of the curving organ. The most convincing evidence supporting the Cholodny-Went theory consists of such data showing that growth on the lighted side of a corn coleoptile decreases during curvature while that on the shaded side increases (Iino and Briggs 1984). This follows the predictions of the Cholodny-Went theory, but not of the alternate theories. Additional support came from experiments of Orbović and Poff (1993) who measured the elongation rates of two opposite sides of hypocotyls of *Arabidopsis thaliana* seedlings during phototropism by using an infra-red imaging system: In first positive phototropism, second positive phototropism and red-light-enhanced first positive phototropism (Hensel 1986), curvature toward the light source was the result of an increase in the rate of elongation of the shaded side and a decrease in the rate of elongation of the lighted side of the seedlings. The phase of straightening that followed maximum curvature resulted from a decrease in the elongation rate of the shaded side and an increase in the elongation rate of the lighted side. These data for the three types of blue-

light-induced phototropism tested in this study and for the phase of straightening are all clearly consistent with the growth rate changes predicted by the Cholodny-Went theory.

In addition, direct measurements of auxin also support the Cholodny-Went theory (Gardner et al. 1974; Iino 1991, 1995). Such direct measurements would be technically quite difficult to make in the hypocotyl of *Arabidopsis thaliana* because of its small size. Coleoptiles of *Zea mays* were successfully used. Iino (1995) investigated whether or not gravitropism and phototropism of *Zea mays* coleoptiles behave as predicted by the Cholodny-Went theory in response to auxin application, decapitation and combination of these treatments. Gravitropism was induced at an angle of 30° from the vertical, and phototropism by a pulse of unilateral blue light. Either tropism of the coleoptile was inhibited by indole-3-acetic acid (IAA), applied as a ring of IAA-lanolin paste to its subapical part, and by decapitation. The dose-response curves for the effects of applied IAA on tropisms and growth of intact coleoptiles as well as the time courses of tropisms, induced in decapitated coleoptiles, could be explained by the three conclusions in the literature: (1) the tip of the coleoptile is the site of auxin production; (2) lateral translocation of auxin in gravitropism occurs along the length of the coleoptile; and (3) lateral translocation of auxin in phototropism occurs in the coleoptile tip. By examining the effects of decapitation made at different distances from the top and of IAA applied to the cut surface of decapitated coleoptiles, it was indicated that auxin is produced in the apical 1-mm zone of an intact coleoptile and that lateral auxin translocation for phototropism takes place in an apical part that somewhat exceeds the zone of auxin production. An indication of auxin redistribution also is the report that a small auxin up-regulated m-RNA (SAUR) is differentially induced during phototropism of transgenic tobacco seedlings (Li et al. 1991).

There is evidence that cytosolic  $[Ca^{2+}]$  may also play a role in the transduction chain for phototropism. Gehring et al. (1990) reported that cells on the shaded side of unilaterally irradiated maize coleoptile tips showed rapid changes in cytosolic  $[Ca^{2+}]$  and pH. There is also some evidence for a role of  $Ca^{2+}$  in gravitropism (see Sect. 3.c). Insight into an initial signal transduction step in phototropism may have been provided by the demonstration that the 100-kDa protein (Hager and Birch 1993) and/or the 120-kDa protein (Palmer et al. 1993a,b), which is/are phosphorylated in response to blue light, is/are related to phototropism in *Arabidopsis thaliana*. Three alternatives, not compatible with each other, need to be considered here: (1) The protein could conceivably be phosphorylated in response to blue light without being involved in any of the physiologically relevant steps in phototropism; (2) Phosphorylation of the protein could be the initial step in the induction of phototropism; (3)

Phosphorylation could be involved in desensitization rather than the induction of phototropism.

At present, there are no data in direct support of any one of these possibilities. Tips of maize coleoptiles, which function as essential light sensors for the phototropic growth reaction, exhibit a rapid blue-light-induced phosphorylation of a plasma membrane-associated 100-kDa protein (Hager and Birch 1993). Characteristics of this reaction are as follows:

1. The functional unit involved in the light-dependent phosphorylation consists of a photoreceptor, a protein kinase and the 100-kDa protein. This complex is only localized in the plasma membrane of tips but not in other parts of the seedling.
2. The photoreceptor is a cryptochrome-like compound.
3. The pH optimum of the light-dependent phosphorylation on isolated plasma membranes is around pH 7.8 whereas the light-independent phosphorylation of other membrane proteins occurs at lower values (pH 6.2).
4. The light-induced in-vitro phosphorylation of the 100-kDa protein is strongly inhibited by the protein-kinase inhibitor staurosporine ( $IC_{50} = 4 \text{ nM}$ ).
5. The P-32-moiety of a P-32-[100-kDa]-protein complex generated after a light pulse with the aid of a membrane-associated protein kinase in the presence of  $[\gamma\text{-P-32}]\text{ATP}$  cannot be removed by a 100-fold higher level of (unlabelled) ATP. This fact indicates that protein and phosphate are covalently connected and that the complex is not a short-lived intermediate.
6. The 100-kDa protein is not identical to the plasma-membrane  $\text{H}^+$ -ATPase, as shown by immunostaining on Western blots.
7. Irradiation-dependent in-vivo phosphorylation of the 100-kDa protein in tips is already saturated by a light pulse of 5 s. In contrast, the dephosphorylation of the protein in the dark is a slow reaction lasting about 30 min.
8. Putatively, the blue-light-sensitive photoreceptor localized to the plasma membrane of the phototropically active tip region of coleoptiles has an autophosphorylatable kinase domain which is able to use ATP or GTP as substrate (Hager 1996).

In dark grown coleoptiles of maize seedlings, blue light was reported by Briggs and coworkers (Palmer et al. 1993a,b) to induce rapid phosphorylation of a 114-kDa protein (see Sect. 2.a) at fluence levels that are sufficient to stimulate phototropic curvature. Phosphorylation in response to blue light can be detected in vivo in coleoptile tips preincubated in  $32\text{-P}_i$  or in vitro in isolated membranes supplemented with  $[\gamma\text{-P-32}]\text{ATP}$  (Palmer et al. 1993a). Phosphorylation reaches a maximum level in vitro within 2 min following an inductive light pulse, but substantial labelling



occurs within the first 15 s. Isolated membranes remain activated for several minutes following an *in vitro* blue-light stimulus, even in the absence of exogenous ATP. Phosphoamino acid analysis of the 114-kDa protein detected phosphoserine and a trace of phosphothreonine. The kinase involved in phosphorylating the protein *in vitro* is not dependent on calcium. The 114-kDa protein itself has an apparent binding site for ATP, detected by incubating with the non-hydrolyzable analogue, 5'-p-fluorosulphonyl-benzoyl-adenosine. This result suggested that the 114-kDa protein, which becomes phosphorylated in response to blue light, may also be capable of kinase activity. Identity of the 114-kDa protein, reported here, with the 100-kDa protein reported by Hager and Birch (1993) remains to be seen.

Blue-light-induced phosphorylation occurs only in the upper portion of the maize coleoptile and is absent from the node and mesocotyl (Palmer et al. 1993b). The specific activity of phosphorylation is highest in the extreme apical portion of the tip, which is also the site of maximal sensitivity to phototropic stimuli. Fluence-response determinations indicated that light dosage levels that stimulate curvature also stimulate phosphorylation. However, the threshold for inducing detectable phosphorylation in maize cannot be matched to the threshold for curvature induction. The recovery of sensitivity to phototropic stimuli after exposure to high fluences of light occurred with kinetics that were very similar to those for recovery of the phosphorylation response after a previous high-fluence light exposure. In addition, wavelengths of light in the blue and near-UV regions of the spectrum that maximally stimulate phototropic curvature also maximally stimulate *in vitro* phosphorylation in maize. The pattern of stimulation matched the absorption spectra of flavoproteins, which have been proposed as candidates for blue-light photoreceptors. Solubilization studies indicated that the 114/116-kDa protein is strongly membrane-bound only at the very beginning of seedling development and becomes more loosely associated in the course of coleoptile growth (Salomon et al. 1996).

Hager et al. (1993) studied redox dependence of the blue-light-induced phosphorylation of the reported 100-kDa protein in maize coleoptiles. Since, under *in vivo* conditions or in a crude homogenate of tips, cytosolic ATP is the phosphate donor for the light-induced phosphorylation of this protein, a subsequent *in vitro* phosphorylation by [P-32]ATP is prevented. However, *in vitro* irradiation of microsomal membranes isolated from non-irradiated tips with subsequent 1-min incubation with [P-32]ATP resulted in a strong phosphorylation of the 100-kDa plasma membrane protein. This process was saturated by a 7-s light pulse ( $200 \mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ ). In the absence of [P-32]ATP the capacity for *in vitro* phosphorylation of the 100-kDa protein after a 30-s light pulse declined slowly within 60 min but could be reconstituted by a new light pulse in the presence of reducing compounds. Moreover, when

plasma membranes which had been stored frozen were used, reducing compounds such as NADH, NADPH, ascorbate, glutathione or dithiothreitol enhanced the light-triggered in-vitro phosphorylation. These compounds were unable to elicit or enhance the phosphorylation in the dark. The authors suggest that the transfer of (blue-light) excited electrons from the chromophore moiety of the receptor to the target (either the 100-kDa protein or the protein kinase) is facilitated when reducing compounds instantly eliminate the positive charge generated at the chromophore. The transferred electrons could finally alter the redox state and/or the conformation of either the 100-kDa protein, rendering it susceptible to the action of a protein kinase, or the protein kinase which would then be capable of phosphorylating the 100-kDa protein (Hager et al. 1993).

In the presence of several thiol reagents at the concentration of 1 mM, light-induced phosphorylation of the 114-kDa protein (see above) in plasma membranes isolated from the tips of maize coleoptiles was investigated by Rüdiger and Briggs (1995). Dark phosphorylation of the protein was not affected, but light-induced phosphorylation was inhibited 50% with iodoacetamide, 75% with N-ethylmaleimide and 93% with N-phenylmaleimide. Previous incubation of the inhibitors with mercaptoethanol abolished the inhibitory activity completely. N-phenylmaleimide showed the same inhibition whether it was applied before or after irradiation of the sample. Involvement of thiol group(s) in processes after photoexcitation is considered.

Hexacyanoferrate (III) ions (HCF) specifically inhibit transduction of the blue-light signal in zygotes of furoid algae (Berger and Brownlee 1994). HCF reduction by *Fucus* sp. zygotes occurred on the outer surface of the plasma membrane at higher rates in blue light than in darkness. These observations suggested that blue-light signal transduction involves a redox chain in the plasma membrane. Low doses of HCF ( $< 50 \text{ pmol} \cdot \text{cell}^{-1}$ ) inhibited photopolarization but not germination, hence uncoupling both processes.

A second blue-light-activated protein (a G protein) has been isolated from the plasma membrane of etiolated pea buds (Warpeha et al. 1992). This might be related to phototropism or to another blue-light physiological response. Items such as this can best be resolved through the judicious use of mutants. Ideally, this would involve the use of tropism mutants and also mutants with alternations in specific components in the G-protein response mechanism. Although a number of phototropism mutants have been described previously (see below), mutants with alterations in possible transduction elements such as the G-protein response mechanism or  $\text{Ca}^{2+}$ -transport elements will be more difficult to obtain.

Hypocotyls of dark-grown seedlings of *Arabidopsis thaliana* exhibit a strong negative gravitropism, which is reduced by red and also by long-

wavelength, far-red light treatments. Light treatments using phytochrome A (phyA)- and phytochrome B (phyB)-deficient mutants showed that this response is controlled by phyB in a red/far-red reversible way, and by phyA in a non-reversible, very-low-fluence response (Poppe et al. 1996). Crosses of the previously analysed phyB-1 allele (in the ecotype Landsberg erecta background) to the ecotype Nossen wild-type (WT) background resulted in a WT-like negative gravitropism in darkness, indicating that the previously described gravitropic randomization observed with phyB-1 in the dark is likely due to a second mutation independent of that in the PHYB gene.

#### d) Mechanism for Measuring Light Direction

A plant in phototropic response detects the direction from which the light is incident and grows toward that light which either is static or moves at certain angular velocity during the day (solar-tracking; Ritter and Koller 1994; James and Bell 1996; Totland 1996). This is accomplished in the absence of any specialized photoreceptor organelle such as an eye. It is likely that the plant has a mechanism for establishing a gradient in the quantum concentration. This light gradient would be translated into a difference in concentration of some photoproduct on the two sides of the plant shoot, and this could result in curvature due to unequal growth rates (cellular elongation rates) of the two sides. Three models have been proposed for establishing a light gradient. One is based on refraction. The second is based on screening. The third is based on absorption dichroism.

In the refraction model, light is refracted at the air/organism interface and thereby focused onto the distal side of the organism. Thus, a higher quantum density is established on the distal side than on the proximal side of the organism. There is considerable evidence to support this model for the very small and rather transparent organs supporting the sporangia of some phototropic fungi (Fukshansky 1993). For example, if the sporangiophore of *Phycomyces blakesleeana* is submerged in oil with an optical index of refraction close to that of the cytoplasm, its ability to grow toward a unilateral light source is lost (Banbury 1959; Kubo and Mihara 1996). Similar experiments have not been successful with plant shoots.

In the screening model, a difference in quantum density is established across the organ by screening of light within the organ. Thus, a higher quantum density is established on the proximal side than on the distal side of the organism. The screening is a consequence of scattering and of absorption. This appears to be the mechanism whereby plants establish a light gradient. The evidence for this has been obtained by manipulating the absorption component of screening. Use of inhibitors and mutants decreasing the visible-absorbing carotenoids has been shown to

cause a decrease in the amplitude of phototropic curvature in maize (Vierstra and Poff 1981; Piening and Poff 1988). These treatments are specific for phototropism. They have no effect on the threshold for phototropism, and they affect the amplitude of curvature only if phototropism is induced by wavelengths that are absorbed by the carotenoids. Therefore, it has been concluded that the carotenoids do not function as phototropism photoreceptor pigments, but rather as screening pigments (Vierstra and Poff 1981; Piening and Poff 1988).

The mechanism of gradient formation through absorption dichroism is observed in plants comprising low cell number but highly light-scattering organelles, where the mechanisms of refraction and screening are not applicable. Fern protonemata, submerged in the aquatic layer, is such a system, and has been studied extensively in the laboratory of Wada. The intracellular localization and orientation of the receptors for the blue-light-induced phototropism in fern protonemata of *Adiantum*, i.e. phytochrome and the blue-light-absorbing pigment, were investigated by combining the techniques of cell centrifugation and of microbeam irradiation with linearly polarized light (Hayami et al. 1992). The phototropic response was induced in the cells even after they had been centrifuged basipetally to spin down the endoplasm from the apical region. When a polarized blue-light microbeam was given to a flank of the apical region of the protonema, the phototropic response after compensation of the phytochrome effect by far-red light was most effectively induced when the polarization plane was parallel to the long axis of the cell. If the phototropic response was mediated through phytochrome alone, polarized light vibrating parallel to the cell axis again was most effective in inducing the response. These results indicate that both the blue-light-absorbing pigment and the phytochrome responsible for the blue-light-induced phototropism in *Adiantum* are confined to the plasma membrane and/or the ectoplasm and that the transition moments of their blue-absorption bands are nearly parallel to the cell's long axis.

#### e) Adaptation

Adaptation processes enable phototropism and other blue-light responses of *Phycomyces* to operate over a 10-decade range of fluence rate ( $1 \text{ nW} \cdot \text{m}^{-2} - 10 \text{ W} \cdot \text{m}^{-2}$ ; Galland 1991). Phototropic latency, used routinely to monitor the kinetics of sensitivity recovery after a step down in fluence rate, can be shortened by application of dim light for 35 min during the early part of the latency period. This light is termed subliminal, because it does not elicit phototropism under these experimental conditions; rather, it exerts its influence on the underlying adaptation kinetics. Fluence rate-response data for this latency reduction, obtained

in the laboratory of Lipson and co-workers at 17 wavelengths of subliminal light from 347 to 742 nm, showed a variety of shapes that could be fit by zero, one or two sigmoidal components, plus a constant term. At most wavelengths, the fluence-rate threshold for latency reduction by subliminal light tended to be well below the absolute threshold for phototropism, indicating that this effect is highly sensitive (Galland et al. 1989; Chen et al. 1993). An action spectrum for the sensitivity of the subliminal light effect, derived from the fluence rate-response curves, shows major peaks around 400 and 500 nm and a broad band from 570 to 570 nm, followed by a steep absorption edge. The sensitivity in the near-UV region, compared with the visible, is very low. The magnitude of the latency reduction also depends strongly on wavelength with a maximum at about 450 nm. The fluence-rate response data and the action spectrum – which is markedly different from that for phototropism and other blue-light responses of *Phycomyces* – indicate the participation of multiple pigments, or pigment states, in the photocontrol of adaptation.

To investigate the influence of calcium on dark adaptation, the phototropic latency method was employed by Sineshchekov and Lipson (1992) with the modification that sporangiophores were temporarily immersed in solutions containing  $\text{CaCl}_2$  or  $\text{LaCl}_3$ . Following such treatment, the time course of bending was found to have two components with distinct latencies and bending rates. After immersion in darkness for 30 min in  $\text{LaCl}_3$  solution or 60 min in a solution of  $\text{CaCl}_2$ ,  $\text{MgCl}_2$  or the calcium chelator ethyleneglycol-bis-(beta-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA), each sporangiophore was adapted to a blue-light beam ( $1 \text{ W} \cdot \text{m}^{-2}$ ) for 45 min by rotation around its vertical axis. Cessation of rotation defined the onset of the phototropic stimulus, at which time the light intensity was reduced by as much as 1000-fold. For a 100 fold reduction (to  $10^{-2} \text{ W} \cdot \text{m}^{-2}$ ), immersion in  $\text{CaCl}_2$  (10–100  $\mu\text{M}$ ) decreased the latency 13 min for the early bending component and 18 min for the late component, whereas treatment with the calcium-channel blocker lanthanum (0.1–11  $\mu\text{M}$   $\text{LaCl}_3$ ) increased the latency 12 min for the early component and 13 min for the late component. EGTA (10  $\mu\text{M}$ ) also had an inhibitory effect, increasing the latency of the first and the second components by 7 and 10 min, respectively. In experiments performed similarly, but without the light-adaptation treatment after immersion, no differences between calcium-treated and control sporangiophores were found. The bending rates of both components showed only a weak dependence on calcium. Finally, experiments with  $\text{MgCl}_2$  (10–100  $\mu\text{M}$ ) were indistinguishable from control experiments, indicating that the divalent cation  $\text{Mg}^{2+}$ , unlike  $\text{Ca}^{2+}$ , had no effect on latency or bending rate. The results show that calcium affects the phototropic latency, and may function in the adaptation processes for *Phycomyces* phototropism.

## f) Cytoskeleton

The F-actin distribution in caulonemal tip cells of the moss *Ceratodon purpureus* was examined by rhodamine-phalloidin staining by Meske and Hartmann (1995), Walker and Sack (1995) and Meske et al. (1996). Gravitropically-growing caulonemal tip cells of the moss possess a distinct alignment of microfilaments in their apices. Axially oriented actin bundles run from subapical regions to the apex where they converge towards a central area of the tip, although bundles are absent from the central area itself, thus forming a collar-like structure. During a unilateral red-light irradiation the actin strands of the apical dome became reoriented towards the irradiated apical flank and still surrounded an area free of microfilaments, the point of prospective outgrowth. This process was closely correlated with the morphological effect of bulging and preceded the light-directed outgrowth. The collar structure was essential for the tubular growth form. In darkness, under the influence of anti-microtubule agents the structure was decomposed, the actin strands drifted along the cell flanks and finally accumulated in randomly distributed areas where further growth took place. The microtubules were not involved in the phytochrome-mediated reorientation of the microfilaments: unilateral red light suppressed the tip-distorting effect of anti-microtubule drugs and restored the collar structure with a pronounced light-directed orientation. The microtubules seemed to be responsible for restricting the reorientation to the cell tip.

## 3. Gravitropism

The only mechanism thus far conceived by which gravity can be measured is its effect on a mass, transforming mass into the attribute of weight. Perception of gravity by a plant is usually divided into several steps, the first of which is susception, the exertion of weight or motion of some mass acted upon by gravity. Perception is considered to have occurred after susception has altered some biochemical/physiological step. The transduction pathway includes all of the processes from susception to the final growth response.

Unfortunately, gravity as an environmental stimulus is extremely difficult to study, because every mass is acted upon by the all-pervasive 1 g of earth's gravity. Thus, the experimenter can manipulate the direction of the gravitational vector and subject the organism to a gravitational acceleration above 1 g through centrifugation, but the minimum gravitational stimulus is 1 g in an earth-based experiment. To avoid this constraint, the clinostat is used in an earth-based experiment to slowly rotate the organism such that the organism experiences a constantly changing gravitational direction. The clinostat does not eliminate the 1 g

force of gravity. It only eliminates its constant directionality (Brown et al. 1976; Briegleb 1992; Wagner 1996). Recently, access to a micro-g environment using parabolic flights (Volkman et al. 1986) and space capsules has become available (Volkman and Tewinkel 1996a,b). Unfortunately, such access is limited at present. Nevertheless, interesting experiments have been performed. The gravitropic curvature of seedlings of lentil (*Lens culinaris* L. cv. Verte du Puy) grown in microgravity and stimulated on the 1 g centrifuge for 5–60 min was followed by time lapse photography in the near weightlessness environment of Spacelab (Perbal and Driss-Ecole 1994). In microgravity, the root tip could overshoot the direction of the 1 g acceleration after bending, whereas roots stimulated on the ground did not reach the direction of the gravity vector. On earth, there is, therefore, a regulation (inhibition of root curvature), which is gravity dependent. In space, the initial rate of curvature as well as the amplitude of curvature varied as a function of the quantity of stimulation. For a given quantity of stimulation, the rate of curvature remained constant for 80 min. The bending has thus a certain inertia, which is linked to the mechanism of differential growth. The presentation time ( $T_p$ ) of the lentil root was calculated by extrapolation to zero curvature of the regression line representing either the initial rate of curvature or the amplitude of curvature at 2 h after the end of the stimulation.  $T_p$  was estimated to 27 and 26 s, respectively. These results confirm the values of  $T_p$  obtained by clinostats, and they also lead to a reconsideration of the causes of the kinetics of root curvature.

Johnsson et al. (1995) conducted a series of gravitropic experiments on *Avena* coleoptiles in the weightless environment of Spacelab. Plants at two stages of coleoptile development were tested. Plant responses were obtained using time-lapse video recordings that were analysed after the flight. The concept of gravitropic dose, the product of the transverse acceleration and the stimulation time, was found well-defined in the acceleration region studied. With the same hardware, tests were done on earth where responses occurred on clinostats. The results did not contradict the reciprocity rule, but scatter in the data was large.

#### a) Susception

Comparable with amyloplasts in higher plants, *Chara* rhizoids have single membrane-bound compartments that appear to function as statoliths. Rhizoids were generated by germinating zygotes of *Chara* in either soil water medium (SWM) or artificial pond water (APW). Differential interference contrast (DIC) microscopy demonstrated that rhizoids from SWM-grown plants typically contain 50–60 statoliths per cell, whereas rhizoids from APW-grown plants contain 5–10 statoliths per cell (Kiss 1994). Rhizoids from SWM are more responsive to gravity than rhizoids

from APW. The growth rate of APW rhizoids was significantly greater than that of SWM-grown rhizoids which suggests that APW rhizoids are not limited in their ability for gravitropic curvature by growth. These APW rhizoids, however, seem impaired in gravity perception.

The arrangement of the microtubule cytoskeleton in tip-growing and gravisensing *Chara* rhizoids has been documented by immunofluorescence microscopy (Braun and Sievers 1994). Predominantly axially oriented undulating bundles of cortical microtubules were found in the basal zone of the rhizoids and co-localized with the microfilament bundles underlying the cytoplasmic streaming. Microtubules penetrate the subapical zone, forming a three-dimensional network that envelops the nucleus and organelles. Microtubules are present up to 5–10  $\mu\text{m}$  basal from the apical cytoplasm region containing the statoliths. No microtubules were found in the apical zone of the rhizoid which is the site of tip growth and gravitropism. Depolymerization of microtubules by application of oryzalin does not affect cytoplasmic streaming and gravitropic growth until the relatively stationary and polarly organized apical and subapical cytoplasm is converted into streaming cytoplasm. When the statoliths and the apical cytoplasm are included in the cytoplasmic streaming, tip growth and gravitropism are stopped. Oryzalin-induced disruption of the microtubule cytoskeleton also resulted in a rearrangement of the dense network of apical and subapical microfilaments into thicker bundles, whereas disruption of the microfilament cytoskeleton by cytochalasin D had no effect on the organization of the microtubule cytoskeleton. It is, therefore, concluded that the arrangement of microtubules is essential for the polar cytoplasmic zonation and the functionally polar organization of the actin cytoskeleton which is responsible for the motile processes in rhizoids. Microtubules are not involved in the primary events of gravitropism in *Chara* rhizoids. A similar conclusion was reached by Monzer and his co-workers in their detailed study of graviperception in the basidiomycete *Flammulina velutipes* (Monzer 1995; Moore et al. 1996). The nuclear density was determined in *Flammulina* with  $1.22 \text{ g} \cdot \text{cm}^{-3}$  (Monzer 1996). Consequently, calculation of the forces exerted by the nuclei showed that nuclear displacement in the submicrometer range already fulfils the physical minimum condition for a statolith. Based on these findings, Monzer (1996) proposed a function of nuclei as statoliths in basidiomycete hyphae.

The actin cytoskeleton is involved in the positioning of statoliths in tip growing *Chara* rhizoids (Braun and Sievers 1993, 1994). The balance between the acropetally acting gravity force and the basipetally acting net outcome of cytoskeletal force results in the dynamically stable position of the statoliths 10–30  $\mu\text{m}$  above the cell tip. A change of the direction and/or the amount of one of these forces in a vertically growing rhizoid results in a dislocation of statoliths. Centrifugation was used as a tool to study the characteristics of the interaction between statoliths and



microfilaments. Acropetal and basipetal accelerations up to 6.5 g were applied with the newly constructed slow-rotating centrifuge microscope (NIZEMI; Friedrich et al. 1996). Higher accelerations were applied by means of a conventional centrifuge, namely acropetally 10–200 g and basipetally 10–70 g. During acropetal acceleration (1.4–6.0 g), statoliths were displaced to a new stable position nearer to the cell vertex (12–6.5  $\mu\text{m}$  distance to the apical cell wall, respectively), but they did not sediment on the apical cell wall. The original position of the statoliths was reestablished within 30 s after centrifugation. Sedimentation of statoliths and reduction of the growth rates of the rhizoids were observed during acropetal accelerations higher than 50 g. When not only the amount but also the direction of the acceleration were changed in comparison with the natural condition, i.e. during basipetal acceleration (1.0–6.5 g), statoliths were displaced into the subapical zone (up to 90  $\mu\text{m}$  distance to the apical cell wall); after 15–20 min the retransport of statoliths to the apex against the direction of acceleration started. Finally, the natural position in the tip was reestablished against the direction of continuous centrifugation. Retransport was observed during accelerations up to 70 g. Under the subsequent 1 g condition, the retransported statoliths showed an up to five-fold increase in sedimentation time onto the lateral cell wall when placed horizontally. During basipetal centrifugations greater than or equal to 70 g all statoliths entered the basal vacuolar part of the rhizoid where they were cotransported in the streaming cytoplasm. Braun and Sievers (1994) conclude that the microfilament system is able to adapt to higher mass accelerations and that the microfilament system of the polarly growing rhizoid is polarly organized.

Infra-red laser traps (optical tweezers) were used by Leitz et al. (1995) to micromanipulate statoliths in gravity-sensing rhizoids of *Chara*. The first step in gravitropism, i.e. susception, can be simulated by optical tweezers. The direct laser microirradiation of the rhizoid apex did not cause any visible damage to the cells. Through lateral positioning of statoliths a differential growth of the opposite flank of the cell wall could be induced, corresponding to bending growth in gravitropism. The acropetal displacement of the statolith complex into the extreme apex of the rhizoid caused a temporary decrease in cell growth rate. The rhizoids regained normal growth after remigration of the statoliths to their initial position 10–30  $\mu\text{m}$  basal to the rhizoid apex. During basipetal displacement of statoliths, cell growth continued and the statoliths remigrated towards the rhizoid tip after release from the optical trap. The authors conclude that the statolith displacement interferes with the mechanism of tip growth, i.e. with the transport of Golgi vesicles, either directly by mechanically blocking their flow and/or indirectly by disturbing the actomyosin system. In the presence of the actin inhibitor cytochalasin B the optical forces required for acropetal and basipetal displacement of

statoliths were significantly reduced and leveled off at a similar low level. Braun (1996) presented a hypothesis for cytoskeletally mediated polar growth in *Chara* rhizoids.

Hodick (1994) puts forward a model to integrate the opposite gravitropic responses of protonemata and rhizoids in *Chara* protonemata. He argues that the statoliths intruding into the apical dome may displace a growth-organizing structure from its symmetrical position in the apex and may thus cause bending by bulging. In the positively gravitropic *Chara* rhizoids only a more stable anchorage of the growth-organizing structure is required. As a consequence, sedimented statoliths cannot dislocate this structure from the vertex. Instead, they obstruct a symmetrical distribution of cell-wall-forming vesicles around the structure and thus cause bending by bowing.

Two major candidates have been proposed as the susceptor in gravitropism of higher plants. The most widely accepted candidate has been the amyloplast. According to the starch-statolith model, these starch-containing plastids physically respond to gravity through sedimentation. This sedimentation activates some cellular response mechanism which initiates the signal transduction chain leading to gravitropic curvature (Sack 1991). Considerable evidence has been advanced supporting the starch-statolith model (Haberlandt 1905, reviewed in Audus 1975; Heathcote 1981; Hillman and Wilkins 1982; Kutschera and Hoss 1995; Baluška et al. 1996a,b; Kiss et al. 1996; Kusnetsov and Hasentstein 1996; Volkmann and Tewinkel 1996b).

An alternative model of gravity perception proposes that the gravity-directed distribution of the weight of the protoplasm could serve as a susceptor. A version of this was originally proposed by Czapek (cited in Wayne et al. 1990), who suggested that the weight of the protoplasm on the lower cell membrane could serve as a mechanism for gravity detection. A more recent model has been proposed by Wayne et al. (1990) based on experimental data from internodal cells of a characean alga that contains no visible statoliths. They present calculations to show that the movement of the plasma membrane induced by protoplasm weight redistribution could produce sufficient potential energy to open ion channels in the plasma membrane. Although this argument is convincing for the mass of the 4- to 6-cm-long cell used by Wayne et al. (1990), it remains to be demonstrated that protoplasm settling in a considerably smaller higher plant cell would produce a detectable change in potential energy. The weight or movement of the cellular component functioning as the gravitropic susceptor must itself be detected by an additional cellular component. Components that have been suggested to fill this role include the endoplasmic reticulum, the cytoskeleton, and stretch-activated ion channels in the plasma membrane.

Mechanosensory calcium-selective ion channels probably serve to detect not only mechanical stress but also electrical, thermal and diverse

chemical stimuli (Pickard and Ding 1993). Because all stimuli result in a common output, most notably a shift in second messenger calcium concentration (Trewavas and Knight 1994), the channels are presumed to serve as signal integrators. Further, in so far as second messenger calcium in turn gives rise to mechanical, electrical and diverse chemical changes, the channels are postulated to initiate regulatory feedback loops. It is proposed by Pickard and Ding (1993) that the channels and the feedback loops play a wide range of roles in regulating normal plant function, as well as in mediating disturbance of normal function by environmental stressors and various pathogens. In developing evidence for the physiological performance of the channel, a model for a cluster of regulatory plasmalemmal proteins and cytoskeletal elements grouped around a set of wall-to-membrane- and transmembrane-linkers has proved useful (Pickard and Ding 1993). An illustration of how the model might operate is presented in Fig. 1.

Two glass microelectrodes were inserted from opposite sides of the root cap into statocytes of *Lepidium sativum* L. immersed in medium with or without cytochalasin D (CD), and intracellular potentials ( $E_i$ ) of statocytes were measured with reference to an earthed electrode in the bathing solution (Sievers et al. 1995). In the absence of CD,  $E_i$  values were  $-160 \pm 2$  mV ( $n = 52$ ) in vertical roots. During the recording of  $E_i$ , the roots were tilted from the vertical by  $45^\circ$  so that in a tilted root one electrode was on the upper side and the other on the lower side; after 5 min the roots were returned to the vertical. At approximately 64 s after tilting (lasting 5–15 s) there was a transient lowering of  $E_i$  (more negative) by an average of 4.7 mV on both the upper and lower sides ( $n = 52$ ). In some cases, this decrease in  $E_i$  was preceded by a transitory increase. Returning the roots to the vertical resulted in a response similar to that obtained by tilting. In roots treated with CD at a concentration of  $3 \mu\text{M}$  for 1 h, the initial  $E_i$  was  $-145 \pm 2$  mV ( $n = 43$ ), and the lowering of  $E_i$  on position change (tilting or returning) was smaller (2.0 mV) in some statocytes ( $n = 50$ ) and higher (8.1 mV) in others ( $n = 14$ ) compared with control roots (without and with dimethyl sulphoxide, DMSO). A higher concentration ( $10 \mu\text{M}$ ) of CD and longer treatment (2 h) further reduced the decrease in  $E_i$  (1.1 mV) on position change ( $n = 26$ ). The observed effects of CD support the hypothesis that statoliths in statocytes are anchored by actin filaments to the plasma membrane and/or to the cortical endoplasmic reticulum. Movement of statoliths during the first step of graviperception may lead to stress changes in actin filaments, affecting the transmembrane potential and also the  $E_i$ .

Immunofluorescence labelling of cortical microtubules was used by Blancaflor and Hasenstein (1993) to investigate the relationship between microtubule arrangement and changes in growth rate of the upper and lower sides of horizontally placed roots of maize (*Zea mays* L. cv. Merit). Cap cells and cells of the elongation zone of roots grown vertically in

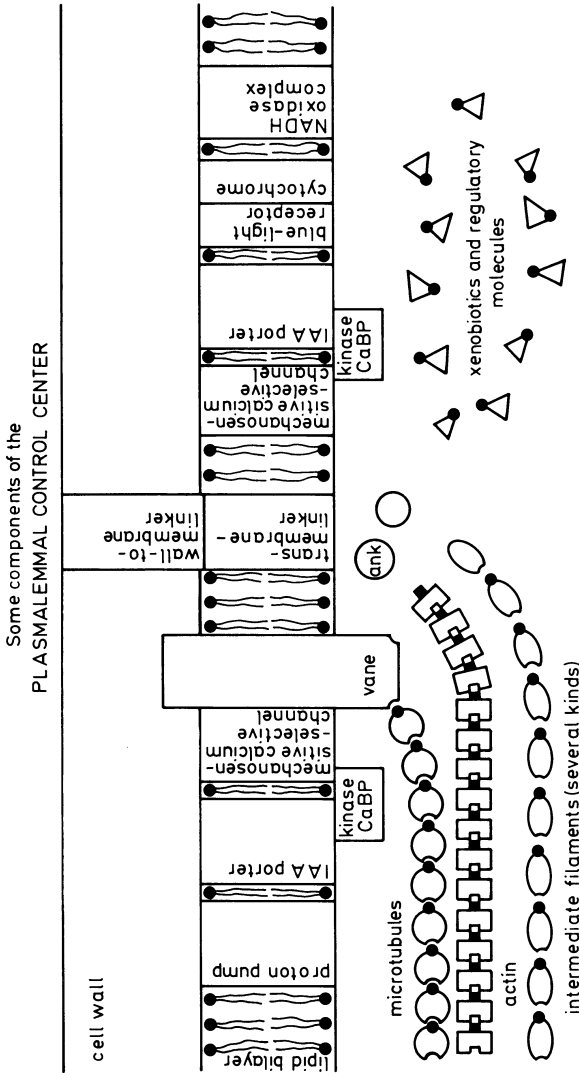


Fig. 1. Some components of the plasmalemmal control centre model of Pickard and Ding (1993). Interconnected clusters of wall-to-membrane-linker and transmembrane-linker form a stretch which transmits force from the cell wall to plasmalemmal mechano sensitive calcium-selective channels. Force can also be transferred to the latter from an internal cytoskeletal network which can anchor to the transmembrane-linker, presumably via anchoring proteins (ank) comparable with ankyrins. A variety of functionally associated plasmalemmal proteins are grouped around the transmembrane-linkers such as proton pump, IAA porter, vane, blue-light receptor, cytochrome and NADH oxidase complex. Some membrane-associated proteins (e.g.  $\text{Ca}^{2+}$ -binding protein-activated kinase) which function and feed-back with the plasmalemmal control center might float freely at the membrane interphase. (Modified from Pickard and Ding 1993)

light or darkness showed microtubule arrangements that were transverse (perpendicular) to the growth direction. Microtubules of cells basal to the elongation zone typically showed oblique orientation. Two hours after horizontal reorientation, cap cells of gravicompetent, light-grown and curving roots contained microtubules parallel to the gravity vector. The microtubule arrangement on the upper side of the elongation zone remained transverse, but the microtubules of the outer four to five layers of cortical cells along the lower side of the elongation zone showed reorientation parallel to the axis of the root. The microtubules of the lower epidermis retained their transverse orientation. Dark-grown roots did not curve and did not show reorientation of microtubules in cells of the root cap or elongation zone. The data indicated that microtubule depolymerization and reorientation are correlated with reduction in growth rate, and that microtubule reorientation is one of the steps of growth control of graviresponding roots. In extended analysis, Blancaflor and Hasenstein in 1995 reported that early stages of gravity-induced curvature occur in the absence of microtubule reorientation, but sustained curvature leads to reoriented microtubules in the outer cortex.

#### b) Site of Perception

In higher plant systems, the root tip has been shown to be the site of gravity-sensing in many classical experiments (for review, see Jackson and Barlow 1981; Poff and Martin 1989). Many authors (see, e.g., Juniper et al. 1966; Pilet 1982) have extrapolated from correlative evidence that the root cap is the site of perception. Some authors (see, e.g., Konings 1968) have even ascribed this function to the columella cells within the root cap. However, the only data specifically directed toward the site of perception ascribe this function to the root tip, which includes the root cap in addition to the zone of division, and possibly part of the growing zone (Poff and Martin 1989).

In hypocotyls and coleoptiles, there is little evidence for a specific region along the shoot axis that is required for gravity sensing. In various studies, auxin (IAA)-induced coleoptile growth has been reported to be closely correlated with an increased occurrence of osmiophilic particles (OPs) at the inner surface of the outer growth-limiting epidermal cell wall, indicating a possible function related to the mechanism of IAA-induced wall loosening. In order to test whether changes in cell elongation rates of upper and lower flanks (UFs, LFs, respectively) during graviresponsive growth are reflected in appropriate changes in the occurrence of OPs, rye (*Secale cereale* L.) coleoptiles, either as segments or as part of intact seedlings, were gravitropically stimulated by positioning them horizontally for 2 h (Edelmann and Sievers 1995; Robinson 1996).

Ultrastructural analyses within the UFs and LFs of the upward-bending coleoptiles revealed a distinct imbalance in the occurrence of OPs. The number of OPs per transverse epidermal cell section of the elongation-inhibited UF on average amounted to twice the number of OPs counted in epidermal cell sections of the fast-growing LF. As an hypothesis, the results suggest that OPs are involved in the mechanism of wall loosening and that temporary growth inhibition of epidermal cells of the UF during upward bending is mediated by inhibition of OP entry into the cell walls. Thereby, more OPs accumulate near the inner surface of the outer wall of epidermal cells of the UF compared with the LF. The region of maximum sensitivity has been directly shown to be present in the most strongly growing region of the elongation zone (Sack 1991).

Ishikawa and Evans (1993) used a video digitizer system to measure changes in the pattern of longitudinal surface extension in primary roots of maize upon application and withdrawal of auxin, and to compare these patterns during gravitropism in control roots and roots pretreated with auxin. Special attention was paid to the distal elongation zone (DEZ), arbitrarily defined as the region between the meristem and the point within the elongation zone at which the rate of elongation reaches 30% of the peak rate. For roots in aqueous solution, the basal limit of the DEZ is about 2.5 mm behind the tip of the root cap. Auxin suppressed elongation throughout the elongation zone, but, after 1–3 h, elongation resumed, primarily as a result of induction of rapid elongation in the DEZ. Withdrawal of auxin during the period of strong inhibition resulted in exceptionally rapid elongation attributable to the initiation of rapid elongation in the DEZ plus recovery in the main elongation zone. Gravistimulation of auxin-inhibited roots induced rapid elongation in the DEZ along the top of the root. This resulted in rapid gravitropism even though the elongation rate of the root was zero before gravistimulation. The results indicated that cells of the DEZ differ from cells in the bulk of the elongation zone with respect to auxin sensitivity and that DEZ cells play an important role in gravitropism.

### c) Elements of the Transduction Chain

As gravitropism is the result of differential growth on opposite sides of a responding organ, it is not surprising that a large number of growth regulators have been associated with this response. These include abscisic acid and auxin (Pickard 1985). There is considerable evidence that auxin has a role in gravitropism (Evans 1991; Trewavas et al. 1992), although there are discrepancies between the timing of auxin asymmetry development and the gravitropic curvature (Firn and Digby 1980). The role of auxins in gravitropism has received strong support from studies of *Arabidopsis thaliana* mutants. Mutations at the *aux1* locus confer

both resistance to exogenous auxin and agravitropism (Mirza et al. 1984). Molecular approaches have yielded additional support for the role of auxin in gravitropism. Small auxin up-regulated mRNAs (SAURs) are more abundant in the side of the gravitropically responding organ that is expected to contain the higher auxin concentration (Li et al. 1991). Together with the observation that a SAUR is found in reduced amounts in a gravitropism-minus *Arabidopsis thaliana* mutant (Gil et al. 1994), this supports some role of auxin in gravitropism.

Colloidal gold-labelled antibody was used by Shi et al. (1993) to localize indole-3-acetic acid in caps of primary roots of *Zea mays* cv. Kys. Gold particles accumulated on the nucleus, vacuoles, mitochondria and some dictyosomes and dictyosome-derived vesicles. This is the first localization of indole-3-acetic acid in dictyosomes and dictyosome-derived vesicles and indicates that dictyosomes and vesicles constitute a pathway for indole-3-acetic acid movement in and secretion from root cap cells. These findings provide cytochemical evidence to support the hypothesis that indole-3-acetic acid plays an important role in root gravitropism.

Primary roots of *Zea mays* (cv. Ageotropic) are non-responsive to gravity and elongate approximately  $0.80 \text{ mm} \cdot \text{h}^{-1}$  (Moore and Maimon 1993). Applying mucilage-like material (K-Y Jelly) to the terminal 1–5 cm of these roots induced graviresponsiveness and slowed down the rate of elongation by 28% (i.e. from  $0.80$  to  $0.58 \text{ mm} \cdot \text{h}^{-1}$ ). Applying K-Y Jelly to one side of the terminal 1.5 cm of the root induced curvature toward the mucilage, irrespective of the root's orientation to gravity. Applying a 2-mm-wide band of the mucilage-like material to a root's circumference 8–10 mm behind the root cap neither induces gravicurvature nor affects elongation significantly. Similarly, applying mucilage-like material to only the root cap does not significantly affect elongation or graviresponsiveness. Gravicurvature of mutant roots occurs only when mucilage-like material is applied to the root/root-cap juncton. Reversing the caps of wild-type and mutant roots produced gravitropic responses characteristic of the root cap rather than the host root. These results are consistent with the suggestion that gravitropic effectors such as benzoxazolinones (see Sect. 2.c) are growth inhibitors that move apoplastically through mucilage between the root cap and root. Baluška et al. (1996a,b) report that  $\text{Ca}^{2+}$  is present in the root cap mucilage in physiologically relevant amounts and can mediate growth responses through asymmetric distribution around the growing root tip.

Studies, that used fluorescent cation indicators to detect free cytosolic calcium, have shown rapid (within about 3 min) increases in calcium concentration in the lower side of a maize coleoptile following a  $90^\circ$  alteration in gravity vector (Gehring et al. 1990). Alterations of calcium levels have been shown in gravistimulated oat coleoptiles, but the changes are less rapid than those reported in maize. The differences in

kinetics could be due to differences in the methods used to detect calcium (Slocum and Roux 1983). In addition, calmodulin has been concluded from inhibitor studies to be involved in gravitropism (Sinclair et al. 1996).

Several investigators have found differential changes in the electrical properties of plants and have suggested that these changes are associated with gravitropism (see, e.g., Bjorkman and Leopold 1987a,b; Ishikawa and Evans 1993; Imagawa et al. 1991; Weisenseel et al. 1992). Mechanosensory calcium-selective ion channels probably serve to detect not only mechanical stress but also electrical, thermal and diverse chemical stimuli. Because all stimuli result in a common output, most notably shift in second messenger calcium concentration, the channels are presumed to serve as signal integrators (Pickard and Ding 1993).

Gating of associations of mechanosensitive  $\text{Ca}^{2+}$ -selective cation co-channels in the plasmalemma of onion epidermis has a strong and unusual temperature dependence (Ding and Pickard 1993). Tension-dependent activity rises steeply as temperature is lowered from 25 °C to about 6 °C, but drops to a low level at about 5 °C. Under the conditions tested (with  $\text{Mg}^{2+}$  and  $\text{K}^+$  at the cytosolic face of outside-out membrane patches), promotion results both from more bursting at all observed linkage levels and from longer duration of bursts of co-channels linked as quadruplets and quintuplets. Co-channel conductance decreased linearly, but only modestly, with declining temperature. The authors propose that these and related mechanosensitive channels may participate in a variety of responses to temperature, including thermonasty, thermotropism, hydrotropism, and both cold damage and cold acclimation.

In summary, it appears likely that gravity is perceived by the plant through the weight of some susceptor (possibly the starch-containing amyloplast, the algal statolith, the basidiomycete nucleus or possibly the entire cell). The weight of the susceptor is detected by its effect on some cellular component (endoplasmic reticulum, cytoskeleton, stretch-activated channel, etc.) The transduction chain may involve changes in the electrical properties of the cell and uses one or more plant growth regulators in roots and shoots to modulate growth rate in the growing zone.

#### 4. Hydrotropism

Hydrotropism has received little attention because of technical and conceptual difficulties. It is difficult at best to imagine studying the sensory response to the most prevalent compound in the plant's internal (and frequently external) environment. Moreover, this is a compound that cannot be eliminated while maintaining physiological conditions. It is clear that maize roots exhibit curvature in response to a differential in



relative humidity (Takahashi and Scott 1993). Roots of peas and maize were exposed to different moisture gradients established by placing both wet cheesecloth (hydrostimulant) and saturated aqueous solutions of various salts in a closed chamber. Atmospheric conditions with different relative humidity (RH) in a range between 98 and 86% RH were obtained at root level, 2–3 mm from the water-saturated hydrostimulant. Roots of Silver Queen maize placed vertically with the tips down curved sideways toward the hydrostimulant in response to approximately 94% RH but did not respond positively to RH higher than approximately 95%. The positive hydrotropic response increased linearly as RH was lowered from 95 to 90%. A maximum response was observed at RH between 90 and 86%. However, RH required for the induction of hydrotropism as well as the responsiveness differed among plant species used; gravitropically sensitive roots appeared to require a somewhat greater moisture gradient for the induction of hydrotropism. Decapped roots of maize failed to curve hydrotropically, suggesting the root cap as a major site of hydrosensing (Takahashi and Scott 1991; 1993; Takahashi et al. 1992).

Takano et al. (1995) and Takahashi (1994) have studied hydrotropism and its interaction with gravitropism in agravitropic roots of a pea mutant and normal roots of peas and maize. The interaction between hydrotropism and gravitropism in normal roots of peas or maize was also examined by nullifying the gravitropic response on a clinostat and by changing the stimulus-angle for gravistimulation. Depending on the intensity of both hydrostimulation and gravistimulation, hydrotropism and gravitropism of seedling roots strongly interact with one another. When the gravitropic response was reduced, either genetically or physiologically, the hydrotropic response of roots became more unequivocal. Also, roots more sensitive to gravity appear to require a greater moisture gradient for the induction of hydrotropism. Positive hydrotropism of roots occurred due to a differential growth in the elongation zone; the elongation was much more inhibited on the moistened side than on the dry side of the roots. The authors suggested that the site of sensory perception for hydrotropism resides in the root cap, as does the sensory site for gravitropism. Furthermore, an auxin inhibitor, 2,3,5-triiodobenzoic acid (TIBA), and the calcium chelator EGTA inhibited both hydrotropism and gravitropism in roots. These results suggest that the two tropisms share a common mechanism in the signal transduction step.

Stinemetz et al. (1996) in the Ageotropum pea mutant determined the threshold time for perception of an osmotic stimulation in the root cap and the time requirement for transduction and transmission of the hydrotropic signal from the root cap to the elongation region. The threshold time for perception of an osmotic stimulation in the root cap was less than 2 min following the application of sorbitol to the root cap. Furthermore, a single 5-min exposure of sorbitol to the root cap fully

induced a hydrotropic response. Stinemetz et al. (1996) also found that transduction and transmission of an osmotic stimulus requires 90–120 min for movement from the root cap to more basal tissues involved in differential growth leading to root curvature.

The site of signal perception and elements in the transduction chain are open problems to date. Gating of associations of mechanosensitive  $\text{Ca}^{2+}$ -selective cation co-channels in the plasmalemma of onion epidermis has a strong and unusual temperature dependence (Ding and Pickard 1993), similar as reported above (see Sect. 3.c).

## 5. Résumé and Outlook

The tools of genetics and molecular biology show great promise for considerable advances in fundamental analysis of tropisms in fungi, and lower and higher plants, if based on an understanding of the physiology and biophysics. It is now abundantly clear that the apparent simplicity of tropisms has been a fantasy. In fact, every model system appears more complex following more detailed analysis study. The available evidence indicates that the phenomenon of tropism is controlled by a complex network leading from a number of sensory inputs to a differential growth response. Evidently, great progress will be made toward a thorough understanding if the true physiological complexities are sufficiently well described to permit careful definition of the experimental system. For example, a mutant screen will always give exactly the mutants being screened for, although these are not necessarily the mutants for which the screen was meant to be designed. Knowledge of the physiology is a prerequisite for intelligent use of genetics and molecular biology, and for the eventual understanding of tropisms at the molecular level.

### Note added in proof

The cloning and sequencing of the gene for the putative photoreceptor protein NPH1 from *Arabidopsis*, currently under way in the Briggs laboratory, could begin to provide a resolution to the long-standing controversy of the identity of the photoreceptor for phototropism (Briggs WR, Liscum E (1997). The role of mutants in the search for the photoreceptor for phototropism in higher plants. *Plant Cell Environ* 20:768–772).

The possibility of reducing or even switching off gravity only arose after the onset of spaceflights. Thirty years after the first microgravity experiments, the background of plant gravitational biology as well as new results and current topics are compiled in the first Supplement to *Planta* Vol. 203 (1997), edited by A. Sievers, B. Buchen and T.K. Scott.

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## **Phloem. Structure Related to Function\***

By Alexander Schulz

### **1. Introduction**

The key tissue for the integration of growth processes within a plant is the phloem. Its highly specialised sieve elements (SEs) form an intimate symplasmic domain for the long-distance transport of assimilates from green source organs to the assimilate consuming or storing sink organs. When functional, the SEs have a greatly reduced cytoplasm and are interconnected by wide sieve pores originating in plasmodesmata, thus forming a low-resistance pathway for translocates. In contrast to the xylem, the driving force for the long distance transport is produced endogenously within the phloem tissue and the conducting cells are vital when functioning, so that changes in assimilate demand can rapidly and flexibly be met.

For understanding the physiology of the phloem, the knowledge of its (ultra) structure is prerequisite, since the transport function is bound to the structural setting. A great number of detailed ultrastructural and physiological papers were published within the last three decades that greatly improved our picture of phloem structure and function. Since, however, comparative approaches were relatively rare, progress went along a somewhat curvy line between confirmations and inconsistencies of physiological and structural data.

The general ultrastructure of phloem elements is today well known, within the restriction given by the fact that electron micrographs are only static images of cells killed for observation. The high sensitivity of the phloem easily leads to artefacts that develop in the time span between dissection of the tissue and the onset of fixation effects. Also, physiological investigations suffer from the sensitivity of the phloem. Changes in temperature, vibrations,  $\text{NH}_4$ -vapour and purposeful wounding immediately inhibit translocation, presumably by closing the sieve pores (Currier and Webster 1964; Anderson and Cronshaw 1969; Lang and Minchin 1986; Galway and McCully 1987; Grusak and Minchin 1989; Pickard and Minchin 1992a–c). Partly, these problems can be over-

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\* Dedicated to Prof. Dr. R. Kollmann on the occasion of his 65<sup>th</sup> Birthday.

come by live observations with a confocal laser scanning microscope (Schulz 1992; Oparka et al. 1994), by transport studies on whole plants with C-11 as marker (Fensom et al. 1977; Minchin et al. 1991) or by nuclear magnetic resonance (NMR) spectroscopy and sophisticated computer evaluations (Köckenberger et al. 1997).

This chapter will treat the structure of phloem elements wherever it appears significant to the function of the phloem. First, the structural background is summarised with regard to the architecture of the phloem and to the ultrastructure of the conducting elements. Emphasis is laid upon those components that discriminate the conducting sieve elements from all other cell types and the role of which is not understood so far. Second, structural adaptations to the different functional tasks of phloem elements are discussed. These adaptations are significant in elucidating the function of SEs which varies in different plant organs. Depending upon the organ, loading, transport or unloading of assimilates prevail over all other tasks.

In this series, structure and special cytology of the phloem were reviewed by Behnke in 1983. For a detailed and well illustrated general survey of and a German introduction to the phloem structure readers are referred to Behnke (1989, 1990a). The comparative structure, induction and development of the sieve elements of the major plant groups, from the algae to the flowering plants, are comprehensively treated in a book (Behnke and Sjölund 1990). The regeneration of phloem (Kollman and Schulz 1993) and the phloem in tissue cultures (Sjölund 1996) were the subject of related reviews in *Progress in Botany*.

In contrast to these reviews showing the variability of the phloem, this chapter will focus on the *convergent* structures in the SEs of the major plant groups because of the author's conviction that a unifying transport mechanism exists in the phloem. Under this assumption, similar (not identical) structures should have evolved in different groups under the stress of adaptation wherever they were advantageous for the function. Convergent structures, thus, emphasise structural prerequisites for the phloem function. Vice versa, a major difference in a decisive structure, as for instance that between the sieve pores of gymnosperms and angiosperms, would jeopardise a hypothesis on the transport mechanism that does not take account of it.

## 2. Phloem Architecture

It is a common experience that the conducting tissues approach the most remote regions in the plant organs. In monocotyledons and dicotyledons the phloem is, moreover, frequently crosslinked by anastomosing strands, thus forming a complex network. Anastomoses occur not only in leaves, but also in stems and fruits. Recently, development and com-

plexity of the network were thoroughly explored by light and electron microscopy in grass leaves (Dannenhoffer et al. 1990; Evert and Russin 1993; Dannenhoffer and Evert 1994; Evert et al. 1996a), in dicot leaves (Ding et al. 1988; McCauley and Evert 1988a,b, 1989), and petiole (Oross and Lucas 1985), and in the shoot of the gymnosperm *Ephedra* (Cresson and Evert 1993). A particular case are the anastomoses of the monocotyledon liana *Dioscorea* which form plexi for the fusion of leaf and bud vascular traces with stem bundles at the nodes and interlink all stem bundles in the internode (Behnke 1990c). In addition, the vascular architecture of the fruit receptacle of red raspberry was exhibited after three-dimensional (3D) NMR microscopy (Williamson et al. 1994).

#### a) Phloem Organisation

Phloem and xylem elements occur in collateral bundles and consist of continuous chains of conducting cells. In a short distance to the apical meristems, the protophloem elements establish the bundles arising from procambial strands. They are subject to extension growth and, thus, become soon obliterated and non-functional. Their loss is compensated for by metaphloem elements subsequently developing from procambial tissue and, in bi- and perennial plants, eventually by cambium-borne secondary phloem elements. Starting at the end of extension growth the vascular tissue is supplemented by supportive tissue. Accordingly, the phloem side of a bundle consists of the conducting SEs, parenchyma and sclerenchyma cells (see Esau 1969; Behnke 1989).

Translocation in the phloem requires (1) the loading of assimilates into the SEs within a source leaf, (2) a continuous conduit between source and sink and (3) the unloading of the translocates in the sink organ. Van Bel (1993b, 1996b) has coined the terms "collection phloem", "transport phloem" and "release phloem" for phloem regions specialised in loading, transporting and unloading, respectively. Derived from their occurrence the main function of protophloem-SEs is to release assimilates towards the near meristem and into immature leaf regions, i.e. they are one-way pathways for assimilate unloading. In contrast, the metaphloem plays a number of functional roles: in mature leaves it is collecting assimilates from the mesophyll, in stem and root it provides the transport conduit and in sink organs it may release assimilates like the protophloem. Where existent, the secondary SEs serve mainly as transport conduit. Secondary SEs do not occur in minor veins of angiosperm leaves, i.e. they are not specialised for loading, although they might be involved in the retrieval of assimilates (Van Bel 1993b). In specific cases, however, secondary SEs are also involved in unloading: in storage organs as, e.g., the tap root of sugar beet, the release phloem consists of

secondary sieve tubes adjacent to the storage parenchyma, both derived from (anomalous) cambia.

#### b) Development and Maintenance of Phloem Architecture

The phloem architecture is maintained and extended by two mechanisms: by indefinite growth of shoot and root and by definite growth of leaves and fruits. Indefinite growth is reflected by the continuous production of protophloem elements close to meristematic apices (see, e.g., Esau and Gill 1973; Thorsch and Esau 1981; Eleftheriou and Tsekos 1982) and metaphloem elements within and beyond the extension zone. This process appears simple compared with the vascular development in leaves which is influenced by the position of the intercalary meristem at the leaf base, on the one hand, and by the shifting zone of the sink/source transition (Turgeon 1989) on the other.

In grass leaves, first protophloem-sieve tubes are formed isolated from the stem vasculature in procambial strands within the leaf. They elongate by the addition of new elements, apically towards the leaf tip and basipetally towards the stem. Metaphloem originates first at the leaf tip and its elongation occurs basipetally (Dannenhoffer and Evert 1994). When photosynthesis starts in the leaf tip, the sink/source transition is also a basipetal process: the border between export and import shifts from the tip region of the leaf blade towards the base (Turgeon 1989). This can be visualised by the import of radioactive assimilates from mature leaves (Ding et al. 1988; Robinson-Beers et al. 1990; Evert et al. 1996a).

During leaf expansion, the only phloem contacts into the leaf blade of maize consist of protophloem-sieve tubes through-connecting the zone of extension and intercalary meristem at the leaf base. Since these become obliterated in the extension zone only one centimetre above, the early metaphloem-sieve tubes present here must function for import in the still largely sink leaf (Evert et al. 1996a). This observation implies that the functional stage of a source leaf – net export – is only reached at leaf maturity, i.e. when the metaphloem at the leaf base is also mature (see Robinson-Beers et al. 1990; Evert et al. 1996a). Prior to that, photosynthates from the leaf tip cannot reach the stem, but might meet the assimilate demand of the intercalary meristem at the leaf base.

Conversely, in the dicotyledonous leaf import and export might occur in parallel. This is possible due to the different (continuous) developmental pattern of the leaf axis and a more differentiated vein hierarchy than in monocots. By comparing the structural maturity of SEs located at the transition zone between import and export regions, evidence was found that the class III minor veins of tobacco leaves are importing assimilates, while the smaller ones, according to their immature SEs, are

still non-functional (Ding et al. 1988). Just beyond this zone the smaller veins – collection phloem *sensu strictu* – contained mature SEs. It can be generalised that in dicotyledonous leaves most of the imported carbon is unloaded by moderately large veins, while the smaller ones are relatively or completely unimportant in this regard (Turgeon 1989).

In the gymnosperm needles, the Strasburger cells mark the exporting (assimilate-collecting) metaphloem and secondary sieve cells (Cresson and Evert 1994; Glockmann and Kollmann 1996). Flanking the phloem part of the needle bundle(s) these Strasburger cells are intimately linked by specific plasmodesmal structures (Glockmann and Kollmann 1996) and form an accessory complex to the sieve cells (see Carde 1973). Position and symplasmic connectivity indicate their involvement in assimilate loading.

The phloem architecture and the developmental pattern of the conducting elements ensure even in huge trees an unbroken transport conduit of less than 1-year-old elements. In order to use this conduit, a given sugar molecule to be transported from a mature leaf to a growing root tip passes several junctions and lateral transitions. It would be loaded into the metaphloem of a collecting minor vein of the smallest order and pass subsequent junctions to higher order veins and to the midrib. The metaphloem of the midrib and the petiole would carry it via the leaf trace to the node of insertion. [Little is known about either the exact structure of the nodal junction or the transfer from the metaphloem into the secondary phloem of woody branches and trunk portions (but see the nodal plexus of monocotyledonous Dioscoreaceae; Behnke 1990c)]. In the secondary phloem the molecule would move till approaching the primary regions of the root. To enter the lateral roots, the molecule had to leave the axial (secondary) phloem and pass metaphloem up to that zone where meta- and protophloem overlap. Lateral transfer into the protophloem would eventually allow the delivery of the molecule towards the meristem. Converse to this simplified picture, it is clear that transport sugars are subject to a multiple lateral exchange so that individual molecules will be metabolised *en-route*. However, it is useful to imagine this pathway, because pathogenic organisms as phytoplasmas (formerly rickettsia- and mycoplasma-like organisms; RLO and MLO) or viruses are carried passively with the photoassimilates, so that spread and progress of systemic infections become predictable (see Leisner and Turgeon 1993).

In conclusion, the described architecture of the phloem is not fixed but very flexible. Elongation of the phloem traces towards the apical meristems by protophloem-sieve tubes and enlargement of the cross-sectional area of the phloem by metaphloem and secondary phloem elements allow a flexible response of the phloem network to lasting changes in assimilate demand. It is pertinent to note that transitions between primary and secondary phloem are of great importance for

connecting new lateral sinks as, e.g., axillary buds, adventitious roots, lateral roots and root nodules. There is some evidence obtained from phloem regeneration that the elongation of the phloem, achieved by the continuous addition of a new element to the terminus of a sieve tube, is induced by sink activity rather than by a surplus of assimilates (Schulz 1986a, 1990b, 1993, 1996a; Kollmann and Schulz 1993; see also Sjölund 1996).

### 3. Ultrastructure of Conducting Elements

#### a) General Considerations

##### α) Evolutionary Trends of Sieve-Element Ultrastructure

If the functional relevance of the ultrastructure of the assimilate conducting cells in the plant kingdom is considered, a tendency towards a *low-resistance conduit* becomes apparent in all plants, starting with the brown algae and mosses. In equipment and low density of the SE-cytoplasm the seedless vascular plants are similar to gymnosperms and angiosperms. There is some variation, however, in the *composition of sieve pores* and their arrangement in sieve areas or plates. Two principles are found: in gymnosperms, the sieve areas are penetrated by numerous sieve pores with diameters generally smaller than 1  $\mu\text{m}$ . An individual sieve pore runs only half-way through the wall common to two SEs. In the middle of this wall, the pore channel approaches an extensive median cavity lined by the plasma membrane. From the cavity the symplasmic continuity is established by another sieve pore (Behnke 1990b; Schulz 1990a). In contrast, in seedless vascular plants and angiosperms the individual sieve pores penetrate the entire wall between SEs, in the end wall often wider than 5  $\mu\text{m}$  (see Esau 1969; Eleftheriou 1990; Evert 1990a). In angiosperms, the end walls (i.e. sieve plates) reflect the evolutionary level of the plant, the parameters being the degree of inclination and its composition (compound or simple, see Esau 1969).

Besides the low-resistance pathway, a second evolutionary trend with functional significance consists in the *degree of symplasmic association* of the conducting SEs to neighbouring parenchyma cells. The intimacy of this association extends from no contact in brown algae (Schmitz 1990), simple and rare plasmodesmata in mosses (Scheirer 1990), single pore/single plasmodesma contacts in seedless vascular plants (Evert 1990a) up to the pore/branched plasmodesmata contacts in gymnosperms (Behnke 1990b; Schulz 1990a) and angiosperms (Eleftheriou 1990; Evert 1990b; for physiology of these contacts in angiosperms see Kempers et al. 1993; Van Bel 1996b; Kempers and Van Bel 1997; Van Bel and Kempers 1997). While in gymnosperms, the associated Strasburger

(i.e. albuminous) cells are ontogenetically unrelated to the SEs (Behnke 1990b; Schulz 1990a); in angiosperms, an intimate contact between the SEs and their associated companion cells is guaranteed by ontogeny: SE and companion cell originate by unequal division from their mother cell.

#### β) Terminology (According to Behnke 1986; Evert 1990a)

The general term for assimilate-conducting cells in all plant groups is "sieve element" (SE). In contrast to the earlier usage of "sieve cell" for lower plants and gymnosperms, this term should be restricted to *gymnosperms* alone, since only within this group structure and development of the sieve cells are sufficiently uniform. This applies especially to the sieve pores which in end as well as lateral walls have the same dimensions. The terms "sieve tube" and, for a single SE, "sieve-tube member" are applicable for all *angiosperms*. Even the primitive SEs in angiosperms share at least two of the three characters: (1) sieve plates in the end walls (defined in having wider pores than the sieve areas in lateral walls; cf. Esau 1969); (2) P-protein (see Sect. 3.e.β); and (3) ontogenetically related companion cells. For *lower plants*, the taxon name can be added to "sieve element" as further specification; e.g. "moss sieve element" (Behnke 1986; Evert 1990a).

#### b) Developmental Stages of SEs

The life of an individual SE, i.e. its differentiation, function and death, starts with the completion of mitosis of its procambial or cambial precursor. In angiosperms, this is the unequal division giving rise to the sieve-tube member and its companion cell. Then, the fate of the future SE is definitely determined. Even under the stress of regeneration, SEs once released from mitosis will not enter mitosis again or differentiate into another cell type. They only might change orientation (Schulz 1986b, 1990b; Kollmann and Schulz 1993). Under the influence of inhibitors as colchicine, preceding divisions might be skipped, but the cell fate is stable (see Hardham and McCully 1982; Eleftheriou 1993a). Companion cells, however, retain their capability for further divisions and redifferentiation. Under regular development, they may subdivide several times (Esau 1969). Under the influence of colchicine, they may differentiate aberrantly into abnormal SEs (Eleftheriou 1993b).

The differentiation of SEs is characterised by the dramatic reorganisation of the cytoplasm and formation of intimate symplasmic connections to the neighbouring cells (to other SEs and to the associated Strasburger or companion cells). The reorganisation involves (1) the loss or disintegration of organelles by the well-known *selective autophagy* of the

SE protoplast, (2) changes in the formation of the endoplasmic reticulum (ER), the widening of symplasmic connections and increase in wall thickness, i.e. the *conversion* of ER, plasmodesmata and the SE wall, and (3) the *acquisition* of specific entities as, e.g., plastidal inclusions and phloem-specific proteins with as yet uncertain functions.

In the context of this chapter, a subjective selection of ultrastructural details is discussed that appears important for the function of mature SEs. Preceding this it shall be emphasised, however, that the relation between structural maturity of an SE and the onset of its functionality has yet to be clarified. It is commonly agreed upon that structural maturity marks the start of transport in SEs. While investigations on regenerating phloem indeed support the view that *effective* translocation only takes place in mature SEs (see Schulz 1987, 1996a; Schulz and Gersani 1990; Kollmann and Schulz 1993), it is still unresolved whether or not a *low-degree* transport precedes this event in developmental stages of SEs. This transport could even evoke the last stages of differentiation (see Sect. 3.c.γ).

### c) Selective Autophagy

Selective autophagy involves the disintegration or loss of the nucleus, dictyosomes, ribosomes and cytoskeletal elements. Thus, from the normal equipment of a meristematic cell, only plastids, mitochondria and specific formations of the ER persist in mature SEs, mostly in a parietal position and in contact with the plasma membrane (plastids and the ER are treated separately).

Mitochondria show only minor changes during differentiation in, e.g., attaining a spherical shape and an electron-translucent matrix. Histochemical tests indicated that the mitochondria of mature SEs contain active cytochrome oxidase, nucleoside phosphatases and acid phosphatases (see Evert 1990b). Activity of the mitochondria could in some plants be prerequisite to SE loading, since the sucrose transporter, located in Solanaceae in the plasma membrane of SEs (Kühn et al. 1997), is energised by ATP-hydrolysis (see Van Bel 1996b).

### α) Nucleus

All mature SEs are enucleate, i.e. they do not have a functional nucleus. In several ferns and mosses and in all gymnosperms, the nucleus disintegrates by pycnosis. The pycnotic remnants persist in mature SEs (Behnke 1990b; Evert 1990a; Scheirer 1990; Schulz 1990a). This was repeatedly documented, and recently also for *Ephedra* which, as a member of the Gnetophyta, is a highly specialised gymnosperm (Cresson and



Evert 1994). During pycnosis, the heterochromatin condenses and is non-functional, since tritiated uridine is not incorporated (Héban 1975) but still stainable with the DNA-specific fluorochrome DAPI (4',6-diamidino-2-phenylindole) (Schulz 1990a). In the other vascular plants, the SE nucleus generally disintegrates by chromatolysis. Both in fluorescence and in electron microscopy (EM), any stainable heterochromatin disappears (Schulz 1987). A pycnotic-type disintegration of the SE nucleus was observed in only the protophloem of some monocots and dicots (Eleftheriou 1990; Evert 1990b).

### β) Ribosomes

Free and bound ribosomes disappear in two steps. First, indication of a change is the decrease of rough cisternal ER and the increase of tubular and stacked forms of smooth ER. Simultaneously with indications of nuclear degeneration and shrinkage of the vacuole, the ground plasma of SEs becomes granular. The granular appearance might be due to separation of the large and small ribosomal subunit and their dispersal over the evacuated cell. When sieve pores are widening, EM-micrographs generally show a clear ground plasma that is free from any ribosomal remnants (Behnke 1989; Behnke and Sjolund 1990). The occurrence in mature SEs of single ribosomes associated with or caught by stacked ER cisternae might be regarded as late disappearing individuals (cf. Eleftheriou 1990).

### γ) Vacuole

Even more important than the disintegration of the nucleus and ribosomes is the disappearance of the vacuole during SE maturation. Its loss seems to be a prerequisite for long-distance transport. It is noteworthy for the development of the pressure-flow hypothesis that Münch (1930) himself saw a problem in a membrane step from the cytosol into the vacuole which, in his conception, was the transport compartment of sugars. Now that the cytosolic compartment is established as transport conduit, the loss of the vacuole appears plausible for the phloem function. However, the term "mictoplasm" (Engleman 1965) is certainly misleading, since extraplasmic compartments still exist in the mature SE (ER lumen, plastidal and mitochondrial outer space). Even if the SE autophagy was introduced by leakage or blending of the content of the vacuole with the cytosol, the thus changed cytosol still belongs to the symplasmic compartment. The disappearance of the vacuole might occur by a pressure reversal between cytosol and vacuole (possibly evoked by the first entry of sugar into the SE incipient in translocation) and a

subsequent shrinkage of the vascular space, or by a change in permeability of the tonoplast or by both (Behnke 1989). The fragmented tonoplast would associate with other remnants of the endomembrane system. This proposal could, however, only be verified with the localisation of tonoplast-specific membrane proteins in mature SEs.

#### δ) Cytoskeleton

The cytoskeleton, in the young stages of SE differentiation frequently encountered, is also decomposed in later stages. Microtubules present from the last division of the mother cell and involved in cell wall deposition (Behnke and Richter 1990; Behnke and Sjolund 1990, Cresson and Evert 1994; Eleftheriou 1994) or secondary divisions of protophloem-SEs (Eleftheriou 1990, 1996) were not detected in later stages of SE differentiation. Actin filaments contributing to plasma streaming and frequently reported from the young primary and secondary elongated SEs of seedless vascular plants and gymnosperms share the fate of the microtubules (Schmitz and Schneider 1989; Evert 1990a; Schulz 1990a, 1992; Cresson and Evert 1994).

#### d) Conversion of ER, Plasmodesmata and SE Wall

##### α) Endoplasmic Reticulum

As mentioned in Section 3.b, ER membranes shed their ribosomes in later stages of SE differentiation. Smooth regions of the ER tend to aggregate with one another or with the plasma membrane and form either stacks of cisternae attached to the plasma membrane or complexes of tubular structures, both being realised in mature SEs or all plant groups (algae exempted; Behnke and Sjolund 1990). Although the functional role of the parietal ER is still far from understood, its particular appearance in mature SEs has led Sjolund and Shih (1983) to coin the term "SER" for "sieve element reticulum".

In gymnosperms tubular ER is aggregated into dense complexes and occurs on sieve areas (Behnke 1989, 1990b; Schulz 1990a; Cresson and Evert 1994) covering and entering the sieve pore orifices. Earlier debates assuming an artificial dragging of the complexes due to the EM preparation and their displacement onto the sieve areas were disproved by confocal laser scanning microscopy of *living* conifer sieve cells (Schulz 1992). According to this approach, the complexes cover not only either side of a sieve area between adjacent sieve cells but also the sieve-cell side of the symplasmic contacts towards Strasburger cells. Purposeful wounding does not evoke a shift of the ER but its swelling, presumably

due to water endosmosis (Schulz 1992). A putative role of the ER complexes in gymnosperms is discussed in Section 4.b.

A typical feature of many dicotyledons SEs – besides the stacks of ER parallel and perpendicular to the plasma membrane – is the continuous, fenestrated ER cisterna covering nearly the entire plasma membrane (Behnke 1989; Evert 1990b). With freeze fracture technique it became evident that this cisternal sheet is fenestrated, i.e. has pores where cytosol and plasma membrane still are in contact (Sjolund and Shih 1983). Its putative role is the sequestering of calcium, as was indicated with histochemical methods by Sjolund (1990a). He discussed also that the ER cisterna forms a micro-environment at the plasma membrane and around mitochondria. This micro-environment might be prerequisite for spatially separating the membrane transporter and ATPase activities from the rapid translocation stream within the sieve tube (Sjolund and Shih 1983). In this "unstirred layer" ATP might be channelled to the proton-pumping ATPase rather than being swept away by the translocation stream (Sjolund 1990a; see also Stitt 1996). This micro-environment might as well be needed for the turnover of membrane and other proteins in mature SEs which should involve an import of (pre-) proteins from the companion cells via the pore/plasmodesma contacts (see Kühn et al. 1997; Van Bel and Kempers 1997).

Still another formation of ER are bundles of 100 or 200 nm tubules found in species of the Nymphaeaceae (Behnke 1996). They appear in connection with normal ER already in nucleate SEs and are densely decorated by particles of 15-nm size. These particles are arranged in linear arrays surrounding the membrane in a ring-like manner and are presumably responsible for the constant diameter of the tubules and their rather rigid shape. In mature SEs the rigid, particle-decorated tubules become mostly invaginated by flexible particle-free ER tubules so that eventually the extraplasmic space is small. Although similar tubules with the same or different diameters are described in literature from contractile vacuoles in *Paramecium*, where the particles consist of  $V_0V_1$  ATPases, or from plant glands, nectaries and microspores (see discussion in Behnke 1996), the specific role of the ER tubules in SEs of Nymphaeaceae remains to be solved.

### β) Plasmodesmata

The maturation of SEs is accomplished by the conversion of plasmodesmata into wide sieve pores. The ultrastructural changes of this process are well characterised and were reviewed comprehensively, among others by Esau and Thorsch (1985), Behnke (1989), Behnke and Sjolund (1990) and Iqbal (1995). In the context of the present chapter, only two points shall be emphasised:

1. The principal difference between SEs of gymnosperms and angiosperms is the obligatory existence of ER membranes in sieve pores of the former plant group (see Behnke 1990b; Schulz 1990a, 1992; Cresson and Evert 1994). The seeming obstructions of angiosperm sieve pores (P-protein filaments or inclusions of burst plastids) are debated and generally agreed upon to occur only as a response to (preparatory) wounding. The ER complexes on gymnosperm sieve areas and in pores not only were shown in well preserved specimens with EM but also demonstrated *in vivo* with a confocal laser scanning microscope (Schulz 1992). The complexity of the pore composition (several half pores from one sieve-area side fuse in a median cavity whereby they achieve contact with the half sieve pores of the other sieve-area side) adds to the comparatively tortuous path for assimilates between individual sieve cells.
2. In all vascular plants the widening of plasmodesmata to sieve pores marks the loss of autonomy of the individual SE. Within the vertical tier of SEs, any active control in the intercellular exchange of molecules vanishes together with the conversion of plasmodesmata. Preceding this event, plasmodesmata between future phloem cells exhibit the regular connectivity as found in parenchyma cells and as was shown by dye injection experiments (Kempers and Van Bel 1997). This would imply a size exclusion limit (SEL) below 1 kDa (for reviews on plasmodesmal structure and function see Robards and Lucas 1990; Lucas et al. 1993; Overall and Blackman 1996). Plasmodesmata, however, do not only sieve solutes with a fixed molecule size, but also have active control over their passage area and the quality of molecules that may pass. Dye injection and marker transport experiments showed that plasmodesmata widen and close according to endogenous demands and that plant viruses are able to abuse the potential of plasmodesmal regulation for their systematic spread in the plant (see Cleland et al. 1994; Waigmann and Zambryski 1994; White et al. 1994; Lucas 1995; Lucas et al. 1995; Schulz 1995; Zambryski 1995; Ehlers and Kollmann 1996; Ding et al. 1996). This potential might rest in the neck region of each plasmodesma where protein bridges normally keep small spaces (2–3-nm width) open for the passage of solutes. Selective binding of endogenous or virus-coded proteins to plasmodesmal proteins could lead to conformational changes (Lucas 1995) so that larger molecules can selectively pass. Only plasmodesmata on the companion cell side of the pore/plasmodesma contacts regularly allow for larger molecules and show SELs of more than 10 kDa (Kempers and Van Bel 1997). Whether the plasmodesmata of these contacts ("pore/plasmodesm units" in Van Bel and Kempers 1997) are also regulated is not determined; if they are, the control in the intercellular exchange between SE and companion cell would be on the companion-cell side.

It is noteworthy that in the nucleate stages of differentiation, sieve pores of the gymnosperm *Metasequoia* have the same neck constriction (40-nm diameter) as the plasmodesmata between Strasburger cells, as shown by high resolution EM (Glockmann and Kollmann 1996). The neck constriction in pore/plasmodesma contacts is preserved on the Strasburger-cell side, but is released on the sieve-cell side later in development (Glockmann and Kollmann, pers. comm.).

#### γ) SE Wall

The lateral cell walls of SEs are generally thicker than those of surrounding cells and often appear double-layered in micrographs. The glistening appearance of these walls in fresh hand sections has led to the term nacre (see Esau 1969). Only sieve areas in lateral and end walls are excluded from thickening. Nacreous walls are defined as primary walls. The composition of the wall layers with respect to cellulosic, pectinaceous and other components is variable in different plant groups. The prominent inner layer of SE walls, sometimes occluding their lumen to more than 80%, may or may not be anisotropic in polarised light, hence consisting of or being devoid of highly ordered cellulose fibrils (Behnke 1989; Evert 1990a,b). In contrast to the thick nacre (primary) walls of SEs in ferns and angiosperm trees, those of Pinaceae exhibit thick secondary walls. A concentric deposition of cellulose fibrils is suggested by their birefringent appearance in polarised light (Schulz 1990a). The terminological difference between primary and secondary walls is given by their deposition before and after the end of extension growth.

The reason for the development of thick SE walls is unknown. It might be speculated that a gradual increase in turgor during SE differentiation promotes a correlated increase in wall deposition. Turgor and the generally helical cellulose orientation in nacreous walls might counteract each other. It could be argued that the degree occupied by the wall compared with the SE diameter is influenced by the preparation of the plant material for microscopy. If the thick nacreous walls are compressed *in vivo*, a turgor release due to the first preparatory cut would evoke a loosening of the inner parts of nacreous walls. However, up-to-date facts to prove or disprove this argument are lacking.

e) Acquisition of Specific Entities with Uncertain Functions:  
SE Plastids and Phloem-Specific Proteins

$\alpha$ ) Plastids

In seed plants, plastids of SEs acquire taxon-specific starch and/or protein inclusions that continue to prove useful in taxonomy (see most recently Behnke 1994, 1995a,b; Behnke et al. 1996). A review about the evolution of forms and types of SE plastids in the dicotyledons was recently given by Behnke (1991a). Six plastid forms with proteinaceous inclusions (crystals and/or filaments) define the P-type and two forms with or without starch grains the S-type. According to this paper, the interrelationship in evolution between all forms could be by a one-step-alteration in loss or gain of starch, protein crystals or filaments. Point of origin could be the Ss-type, i.e. plastids containing grains of the typical SE starch which is spherical, rich in amylopectin and stains reddish with iodine (Behnke 1991a).

Nothing is known about the function of these inclusions. The specific form is stable within the plant and neither starch nor protein become metabolised in mature elements. Transcription and translation/synthesis of these inclusions are obviously part of the genetic programme for SE differentiation, since species-specific inclusions are realised even under stress, i.e. in regenerating phloem (Behnke and Schulz 1983; Kollmann and Schulz 1993). In these and related cases (Dörr 1990) conversion of amyloplasts into SE plastids involves the decomposition of the amyloplast-type starch grains. It is noteworthy that Wang et al. (1995) have identified a phloem-specific  $\beta$ -amylase using a monoclonal antibody raised against SEs from callus cultures. They discuss that this amylase might prevent a buildup of large amyloplast-type starch grains which would impede phloem translocation. A high level of sucrose as normal in sieve tubes would in parenchyma cell plastids lead to the formation of large amyloplast-type starch grains as normally found in storage tissue or as transitory starch in chloroplasts (for interdependence of sucrose level and starch metabolism see, e.g., Geigenberger and Stitt 1991).

The biochemical characterisation of plastidal proteins in SEs is to rank high on the research agenda in order to uncover their functional significance. If one considers the complex pathway of their synthesis, it is hard to believe that plastidal proteins are without importance. It can be assumed that they are encoded by the nuclear, not the plastidal, DNA, translated on cytosolic ribosomes and imported as (pre)proteins(?) across the plastidal envelope membranes (for a recent review on protein import into plastids see Lübeck et al. 1997), although this is experimentally not confirmed yet. Except for Pinaceae, where a phloem-specific protein could be immunolocalised to the plastid crystals (Schulz et al. 1989), isolation of plastidal proteins has not succeeded yet. In the Pinaceae,

purification and partial characterisation indicated lectin-like properties of the isolated protein (Schulz et al. 1989).

### β) Phloem-Specific Proteins

Phloem proteins are specific components of angiosperm SEs. Those having a characteristic ultrastructure in situ can be grouped as (1) dispersive P-proteins (P-protein tubules and filaments) and (2) non-dispersive crystalloids (P-protein bodies). The latter may be of cytoplasmic (e.g. in Fabaceae) or nuclear origin, with the latter only released into the cytoplasm when the nuclear envelope collapses during chromatinolysis. Nuclear protein crystals without known function are specific for one family of the monocots and three of the dicots (Behnke 1991b, 1994, 1995b). A survey of non-dispersive P-proteins in woody angiosperms was given by Behnke (1991b), with special regard to their taxonomic significance.

In the sieve-tube exudate a wide variety of proteins could be identified biochemically. Their size distribution after SDS-PAGE is genus-specific (Cronshaw and Sabnis 1990). Only in cucurbits was it possible to relate a structural protein to a protein band (PP1; 90–100 kDa) separated by gel electrophoresis. Kollmann and co-workers were able to solubilise P-protein filaments and reversibly aggregate them into filamentous structures that resembled the native structure (Kollmann et al. 1970; Kleinig et al. 1971; Kollmann 1980). As dimer, a second cucurbit phloem protein (PP2; 23–28 kDa) with haemagglutinating properties binds covalently to PP1. Ultrastructure and biochemical properties of the phloem proteins were reviewed by Cronshaw and Sabnis (1990) and Sabnis and Sabnis (1995).

*Biochemistry of Soluble Phloem Proteins.* Biochemical research on phloem proteins has been extended since 1990 and, apart from cucurbits, phloem sap has also been analysed from *Ricinus*, *Triticum* and *Oryza* (Fisher et al. 1992; Sakuth et al. 1993; Ishiwatari et al. 1995). These studies depicted a high number of soluble proteins in the phloem sap which was either harvested by bleeding from cuts (*Ricinus*) or aphid stylets (*Triticum* and *Oryza*). These soluble proteins show a considerable turnover when S-35 (cysteine/) methionine was fed (Fisher et al. 1992; Sakuth et al. 1993). The turnover takes place in the companion cells as was confirmed with LM micro-autoradiography by Fisher et al. (1992). In addition to the exchange of soluble, up to 40-kDa large proteins between SE and companion cells, the authors postulate a source/sink movement of these proteins in the sieve tubes that might be indicative of interactions between source and sink.

After isolation and purification, important progress could be achieved by identifying a few of the soluble proteins. Ubiquitin and chaperons were detected in *Ricinus* and involved in the protein turnover in the enucleate SEs (Schobert et al. 1995). In addition, peptidyl-prolyle *cis-trans* isomerase was found that as protein-folding enzyme might also participate in the protein turnover between SE and companion cell (Gottschalk et al. 1996). By molecular cloning Ishiwatari et al. (1995) identified thioredoxin h as one of the major proteins in *Oryza* phloem sap. They discuss the role of this disulphide-reductase as repair enzyme for important proteins in sieve tubes (transporters and ATPase), the replacement of which by companion cell turnover would take more time (see also Raven 1991).

**Biochemistry of Structural Phloem Proteins.** With regard to structural P-proteins considerable progress has been made by two approaches: isolation of SEs and molecular biology of the sieve-tube exudate. Sjölund (1990b, 1996) isolated SEs of *Streptanthus* callus and raised monoclonal antibodies against their proteins. As was mentioned in Section 3.e.α, one clone was specific for a phloem-specific  $\beta$ -amylase with a Mr of 57 kDa (Wang et al. 1995). Three clones were shown to bind to a 89-kDa band from *Streptanthus* phloem-containing extracts in Western blots. They recognised specifically the filamentous P-protein of *Arabidopsis* in immunogold EM (Tóth and Sjölund 1994) and cross-reacted in different degree with more or less related genera (Tóth et al. 1994). No immunobinding was found in grasses which is consistent with the ultrastructural evidence that Poaceae lack P-protein filaments (see Behnke 1981).

Thompson and co-workers were able to clone several cDNAs from the phloem exudate of *Cucurbita maxima* corresponding to PP1 (filamentous protein) and PP2 (phloem lectin). PP1 is encoded by an mRNA of about 2500 nucleotides and PP2 by an mRNA of 1000 nucleotides (Bostwick et al. 1992). The PP2 mRNA was localised by in situ hybridisation in the companion cells of the bicollateral bundles and the extrafascicular phloem of pumpkin. Recombinant proteins encoded by the open reading frame of the PP2-cDNA demonstrated the chitin-binding characteristic of a PP2 subunit (Bostwick et al. 1992). Sequence analysis of the PP2 cDNA showed an open reading frame of 654 base pairs encoding a cytosolic polypeptide of 218 amino acids with a calculated molar mass of 24.5 kDa (Bostwick and Thompson 1993) fitting nicely to the biochemically derived 25 kDa of Read and Northcote (1983). At the nucleic acid and deduced amino acid levels, this PP2-cDNA (*C. maxima*) and that of *C. pepo* (Wang et al. 1994) were 96% identical (Bostwick et al. 1994). Further molecular analysis revealed three genomic clones of the PP2. One of these contained two PP2 genes with a high sequence homology (Bostwick et al. 1994). Comparison of nine different species of the



genus *Cucurbita* demonstrated a high level of PP2-DNA polymorphism contrasted by a single mRNA of 1 kb in all species (Bostwick et al. 1994). Further investigations of this group (Dannenhoffer et al. 1997) showed that expression of the phloem lectin in *Cucurbita* is developmentally regulated. In seedling development the accumulation of the PP2-mRNA in hypocotyls precedes that of the protein by about 1 day. In contrast to Nuske and Eschrich (1976), there was no indication of a high turnover of the phloem lectin, since the labelled protein amount was stable also when the PP2-mRNA decreased (Dannenhoffer et al. 1997). According to indirect immunogold labelling, the lectin is synthesised in the companion cells and then transferred (via the pore/plasmodesma contacts) into the SEs. Young stages of SEs, although containing filamentous P-protein, did not show lectin labelling (Dannenhoffer et al. 1997).

*The Possible Movement of Structural Phloem Proteins.* Although the lectin property of the PP2 of cucurbits has given rise to speculations about its involvement in pathogen resistance or as an anchor for the persisting organelles in conducting SEs (see Cronshaw and Sabnis 1990; Sabnis and Sabnis 1995), there is no evidence for one of these roles. However, in grafting experiments the species-specific pattern of the protein bands after SDS-PAGE allowed the detection of the movement of structural P-proteins or their precursors in the phloem. When *Cucumis sativus* was grafted on *Cucurbita ficifolia*, in the exudate of the scion several additional proteins appeared that corresponded exactly to that of the stock, including the PP1 and PP2 (Tiedemann and Carstens-Behrens 1994). Subsequently, a developmental analysis showed that these proteins appear only after interspecific phloem bridges were established (Golecki and Kollmann 1996). The authors raised the exciting question whether the structural proteins themselves are transported from the stock into the scion, or whether different factors are responsible. This question poses several problems:

1. The sieve pores would form a bottle neck in the transfer of entire structural proteins in the phloem. Those at the graft interface generally are smaller than regular ones (Tiedemann 1989; Kollmann and Glockmann 1990). In addition, the fate of translocated proteins in the target tissue is totally unclear. A putative proteolytic digestion is questioned by the existence of several protease inhibitors in the phloem exudate (Chino et al. 1991; see also Sakuth et al. 1993). In the case that the protein recycling machinery of the companion cells is used, the proteins have to pass the sieve pores/plasmodesma contacts. If this machinery can not be used (as, e.g., in the protophloem where companion cells are often lacking), proteins would accumulate and inhibit translocation.
2. A second possibility is the transport of mRNA across the graft interface. Evidence is accumulating that not only viral (Lucas et al. 1993)

but also endogenous nucleic acids may move through plasmodesmata (see Lucas 1995; Lucas et al. 1995). A companion-cell transcribed mRNA would then, while approaching the long-distance conduit via the pore/plasmodesma contacts, be swept away by the assimilate flow and have access to companion cells of the scion where it can be translated. The products could thus – after a further passage of the pore/plasmodesma contact – become a component of the sieve-tube exudate.

3. Eventually, as a third possibility, a yet unknown (transcription) factor might be translocated and switch on a quiescent (conserved) gene that is only under these circumstances expressed. Transport of a putative long-distance signal molecule was discussed by Lucas et al. (1996).

Whatever the reason for the migration of proteins, mRNAs or unknown factors between the graft partners, its identification will elucidate important phenomena such as the incompatibility between species and the mechanism of phloem transport.

#### f) Implications Arising from the Specific Ultrastructure of SEs

The abandonment of most organelles and the parietal position of the remaining ones appear as an advantageous process promoting a low-resistance pathway for assimilates. It leads, however, to the loss of autonomy of SEs and their dependency on associated cells for most vital processes. Compared with the other highly specialised, enucleate but living cell type in eukaryotes, the mammalian erythrocyte, SEs have a much higher longevity. This might be due to the enhanced activity of the associated cells in energy metabolism, turnover of proteins and membrane constituents and wound responses. The importance of the associated cells is indirectly supported by the comparison of protophloem with metaphloem and secondary SEs. Protophloem elements are rarely associated with companion or Strasburger cells and obliterate already after a few days (Esau 1969; Behnke 1989; Cresson and Evert 1994). Metaphloem and secondary SEs generally have this association and show a longevity of up to 10 years in dicotyledons (see Evert 1990b) and up to 50 years in palms (Parthasarathy 1980). According to Raven (1991), the protection of the long-lived SEs from  $O_2$  and radicals demands effective mechanisms of damage avoidance and damage repair, including the presence of scavenging mechanisms such as glutathione, peroxidase and ascorbate. Raven (1991) also discussed the possibility of replacement of damaged polypeptides in enucleate SEs by symplasmic transfer from companion cells.

So far, the role of SE plastids and structural P-proteins remains uncertain. A decisive role in translocation for the latter can, however, be ex-

cluded, since neither SEs of gymnosperms nor grasses exhibit structural P-proteins (Behnke 1981, 1990b; Schulz 1990a).

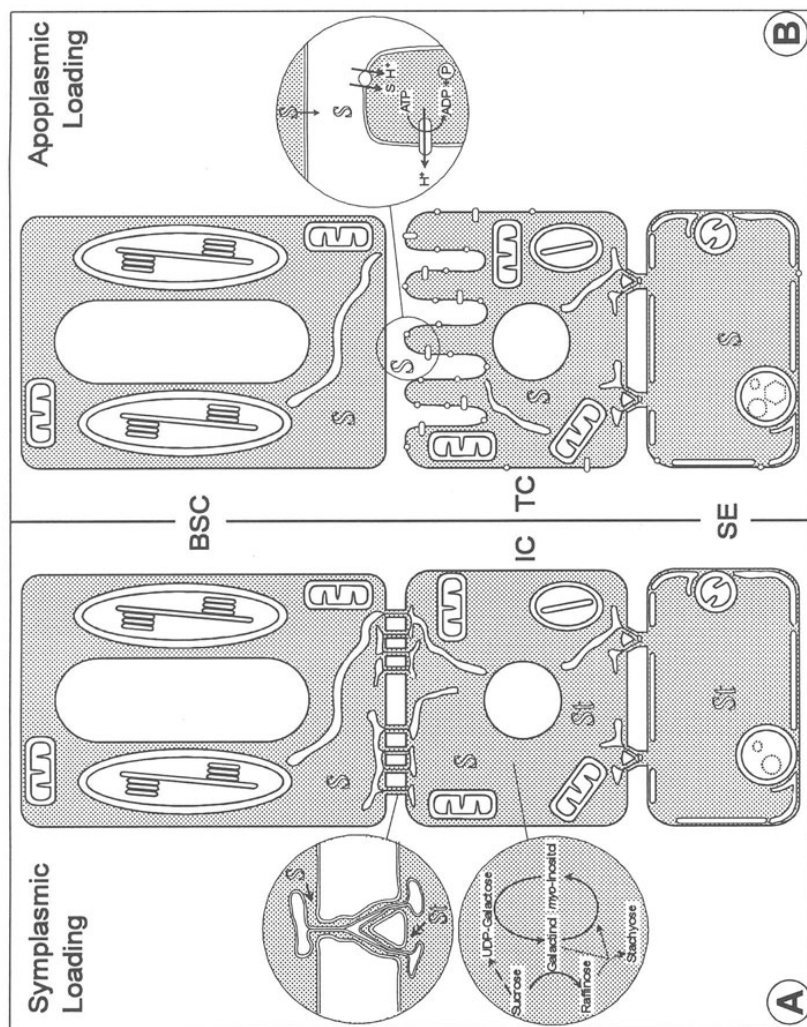
#### 4. Structural Adaptations to the Functional Tasks of Phloem

In the preceding section the general structure and equipment of the conducting elements of the phloem were described. Now it is to be discussed whether the functional tasks of the phloem – loading, transport and unloading – are accompanied by special structural adaptations of the conducting cells. Because of the coverage in literature only seed plants are considered. In these plants, SEs and neighbouring cells associated with them (Strasburger and companion cells) form a functional unit. In this chapter, phloem loading and unloading shall be used in the narrow sense and refers only to the uptake and release of assimilates into and from this functional unit (see Oparka 1990; Van Bel 1993a). Neither the transfer of assimilates from mesophyll to bundle-sheath cells (cf. Van Bel 1996a) nor the "post-phloem transport" after SE unloading (see Wang and Fisher 1994a,b; Patrick and Offler 1995; Patrick 1997) shall be considered here.

##### a) Collection Phloem

The collection phloem in leaves is formed by the metaphloem of minor veins. In the past, the pathway of phloem loading was a matter of some controversy. Evidence is now accumulating that at least two principal alternatives are realised in the plant kingdom: the symplasmic and the apoplasmic mode of phloem loading. (For reports on the taxonomic distribution and reviews of the putative ecophysiological role of the mode of phloem loading, see, e.g., Gamalei 1989; Van Bel and Gamalei 1992; Van Bel 1993a; Eschrich and Fromm 1994; Gamalei et al. 1994.) Structurally, there are clear parameters that allow us to distinguish between: (1) companion plants with a high degree of symplasmic connectivity between the SE/(CC) complex and the rest of the leaf symplast, and intermediary-type companion cells, indicative of a *symplasmic* mode of loading (Fig. 1A); and (2) plants with few or virtually no plasmodesmata between the SE/CC complex and the rest of the leaf symplast, and transfer-type companion cells (i.e. with a wall labyrinth), indicative of an *apoplasmic* mode of phloem loading (Fig. 1B). However, a large number of plants are not easily assigned to either category, and the decision about the mode of loading can only be made after functional tests (see Van Bel et al. 1994, 1996; Komor et al. 1996).

Major progress in understanding phloem loading came in the last 7 years with two approaches: the analysis of the substructure of plasmodesmata, their distribution and frequency between mesophyll cells and the minor vein phloem by EM, and the isolation and characterisation of membrane transporters and of the plasma-membrane-bound ATPase by molecular biology. Both approaches were linked with one another by the immunocytochemical localisations accomplishing the



**Fig. 1.** Two structurally distinct modes of phloem loading. **A** Symplasmic loading indicated by numerous plasmodesmata between bundle-sheath cell (BSC) and intermediary cell (IC) and by formation and accumulation of tri- (raffinose) and tetrasaccharides (stachyose; St) in this cell. Due to the size exclusion limit of branched plasmodesmata between IC and BSC (inset), polymers are trapped in the SE/CC complex and can only move via pore/plasmodesma contacts into the sieve-element system (SE). **B** Apoplasmic loading is reflected by symplasmic isolation of the SE/CC complex and by transfer-type companion cells (TC) with wall labyrinth. Sucrose (S) released from the BSC into the apoplast is actively taken up by a sucrose/proton cotransporter which is energized by ATP hydrolysis. Sucrose accumulating in the TC also approaches SEs via pore/plasmodesma contacts. Sucrose transporter proteins may occur and be active in TC and/or SE.

picture and giving final evidence for the function of intermediary cells in symplasmic loading (Holthaus and Schmitz 1991; Beebe and Turgeon 1992) and of either companion cells or SEs in apoplasmic loading (Bouché-Pillon et al. 1994; Stadler et al. 1995; DeWitt and Sussman 1995; Stadler and Sauer 1996; Kühn et al. 1996, 1997).

### $\alpha$ ) Symplasmic Loading

A prerequisite for symplasmic loading is the existence of a sufficiently high number of plasmodesmata linking the SE with the rest of the leaf symplast (see the open "type 1" of minor veins; Gamalei 1989). The absence of plasmodesmata self-evidently precludes symplasmic loading. Plasmodesmograms are a useful tool to visualise the symplasmic connectivity schematically (Van Bel et al. 1988; Botha and Van Bel 1992; Van Bel and Oparka 1995). These authors, however, emphasise also that the frequency of plasmodesmata and the rate of symplasmic exchange must not necessarily coincide, since the functional diameter of plasmodesmata might be variable.

At first sight, however, symplasmic loading via plasmodesmata as such appears contradictory. Accumulation of sucrose (or other transport sugars) in the transport conduit occurs up-hill and, thus, requires a membrane step with a transporter protein or another driving force. A membrane step is not given when the substructure of plasmodesmata is considered. The symplasmic route from cell to cell is given by the cytoplasmic sleeve between the plasma membrane and the desmotubule. This route is tortuous but without membrane limitations or any indication of a selective transport mechanism for small solutes (Robards and Lucas 1990; Ding et al. 1992; Botha et al. 1993; Overall and Blackmann 1996). The extraplasmic route offered by plasmodesmata is the desmotubule, which is part of the ER compartment but in general appressed to such a degree that only single water molecules can pass (Overall et al. 1982).

*Polymer-Trap-Hypothesis.* An elegant solution to the problem of loading across plasmodesmata was given by Turgeon (1996) with the "polymer-trap hypothesis" of symplasmic phloem loading (Fig. 1A). In brief, this hypothesis applies to those plants which translocate sugars of the raffinose family of oligosaccharides, as, e.g., the cucurbits. According to the model, the accumulation of carbohydrates in the SE/CC complex is active in that raffinose and stachyose are synthesised here. Enzymes involved in raffinose and stachyose synthesis from sucrose (Fig. 1A) were immunolocalised to the intermediary-type companion cells by Holthaus and Schmitz (1991) and Beebe and Turgeon (1992). Raffinose and stachyose are too large to diffuse back into the mesophyll as was indicated by

measurement of the carbohydrate concentration in microdissected tissue (Haritatos et al. 1996).

As mentioned above, plant species translocating raffinose and stachyose in the phloem are distinguished by intermediary-type companion cells in collecting phloem. Ultrastructurally, intermediary cells contrast to normal companion cells by having many small vacuoles and numerous plasmodesmata that link to bundle-sheath cells (Turgeon et al. 1975, 1993; Turgeon and Beebe 1991). These are predominantly branched on the companion-cell side as shown, e.g., for melon and squash. Branching occurs concomitantly with the sink/source transition of the leaf (Volk et al. 1996). According to this paper, ordinary companion cells were not labelled by antibodies against galactinol synthase. It is noteworthy that in *callus* phloem of squash, plasmodesmata connecting to SEs and companion cells are rare (Lackney 1991). By plasmolysis studies, Lackney and Sjolund (1991) demonstrated that callus-SEs accumulate considerably more solutes than companion and parenchyma cells. This contrasts with a symplasmic loading and the proposed polymer trapping where the osmotic potential in intermediary cells should at least equal that of SEs. However, callus phloem is obviously not specialised for loading, and its companion cells differ from intermediary cells (cf. Sjölund 1996). Also, ordinary companion cells of minor veins appear incompetent in polymer-trapping, since they were not labelled with antibodies against the key enzyme galactinol synthase (Beebe and Turgeon 1992).

Flora and Madore (1996) extended the polymer-trap hypothesis and added polyol-translocating species to the symplasmic loading plant species since they are similarly insensitive to p-chloromercuribenzenesulphonic acid (PCMBs) treatment (see also Van Bel et al. 1994). The companion cells in minor veins of mannitol or sorbitol translocating species do not have intermediary-cell characters, but they do have very peculiar plasmodesmata, "H"-shaped in *Petroselinum*, between companion cells and bundle-sheath cells. Flora and Madore (1996) conclude that symplasmic phloem loading may predominate in those plant species in which a combination of sucrose and any other carbohydrate, including the polyols, is translocated.

*Pore Loading of SEs* (cf. Woording 1974). The contradictory up-hill transport via plasmodesmata was circumvented also by a model presented by Gamalai et al. (1994; see also Van Bel 1996a). According to this model, photosynthates would move from mesophyll into SEs exclusively in the extraplasmic endomembrane compartment, with intercellular transport occurring via the plasmodesmal desmotubules. Figure 11 of their paper (Gamalai et al. 1994) indicates a widened desmotubule in the "functional stage". However, evidence from pea roots – where plasmodesmata responded to experimental treatment with an increased sucrose

flux – conversely showed an unchanged desmotubulus and a widened plasmodesmal sleeve (Schulz 1995). Moreover, functional studies on the pore/plasmodesma contacts between companion cells and SEs indicated (compared with "standard" plasmodesmata) an increased SEL for *cytosolic* markers, i.e. markers using the plasmodesmal sleeve (Kempers et al. 1993; Kempers and Van Bel 1997). Gamalei et al. (1994) do not answer the problem of either how sucrose, synthesised in the cytosol of the mesophyll, is taken up by the ER or how and where the sucrose is released from the ER into the sieve-tube lumen. The pore/plasmodesmata contact of angiosperms – considered by Wooding (1974) to offer a putative pathway for "pore loading of sieve tubes" – contains desmotubules on the plasmodesmal (companion-cell) side that at maturity end blindly within the median cavity in the middle of the wall and are not continuous through the sieve-pore (SE) side (Esau and Thorsch 1985).

The situation might be different in gymnosperms. Pore/plasmodesma contacts between Strasburger cells and sieve cells are penetrated by branched ER tubules within the plasmodesmal *and* the sieve-pore side (Glockmann and Kollmann 1996). Moreover, all sieve areas are covered by a complex of ER tubules independent whether they link two adjoining sieve cells or a sieve cell with a Strasburger cell, as was confirmed in living phloem by confocal laser scanning microscopy (Schulz 1992). According to the sequential structure, assimilates are collected by passing outer, middle and inner Strasburger cells, the latter being the only ones in contact with sieve cells (Carcade 1973). Specific, dome-shaped wall portions provide numerous branched plasmodesmata between the Strasburger cells (Glockmann and Kollmann 1996). The dilation of the desmotubules in these plasmodesmata is indicative of micro-osmotic differences between sleeve and ER lumen. It might be speculated that these differences indicate participation of the ER compartment in assimilate loading. A scenario is certainly feasible where membrane transporters in the Strasburger cell ER would differentially "load" this compartment, and where the release into the SE lumen might be via the enlarged surface of the ER complexes. Differential regulation of assimilate gradients in the cytosolic and ER compartment could occur by the antagonistic closure of sleeve or desmotubule in the neck region of Strasburger cell plasmodesmata. (It is obvious that phloem loading in gymnosperms urgently demands functional studies, since the growth of the entire tree depends upon the phloem loading in needles. The importance of softwood production in the northern hemisphere certainly justifies major research efforts.)

In conclusion, symplasmic loading is structurally indicated by particular plasmodesmal contacts between bundle-sheath cells and the cells associated with SEs. Depending on the species, the associated cells are ordinary companion cells, intermediary cells or Strasburger cells. Plasmodesmal contacts are generally numerous and multiply branched on

the side of the associated cells or "H"-shaped. The final step in symplasmic assimilate transfer is the passage of the pore/plasmodesma contact from the associated cell into the SE. Except for gymnosperms – where the mode of loading deserves further investigation – the symplasmic loaders are supposed to translocate a *combination* of sucrose and either sugars of the raffinose family or sugar alcohols.

### β) Apoplastic Loading

Apoplastic loading of the phloem involves existence of membrane transporters in the plasma membrane of the SE/companion cell complex. In general, these co-transport sucrose and protons from the apoplast into the SE/CC complex. This active transport is energised by plasma membrane ATPases (Fig. 1B; for review see Delrot 1989; Van Bel 1993a; Komor et al. 1996). The main transport sugar in apoplastic loaders is sucrose.

*Absence of Plasmodesmata.* Structural evidence for the apoplastic mode of phloem loading is only given by the *absence* of plasmodesmata between bundle-sheath cells and the SE/CC complexes in minor veins. In several plant species the transfer-type companion cells are a further structural hint (see "type 2b" of minor veins; Gamalei 1989). Except for their common wall with the SE, these companion cells have elaborate wall ingrowths and, thus, a greatly increased surface area of the plasma membrane. Size and number of wall ingrowths are positively correlated to the photon flux density (Wimmers and Turgeon 1991). The sucrose flux across the plasma membrane of transfer cells was calculated at 14.3 and 30 pmol · cm<sup>-2</sup> · s<sup>-1</sup> (Gunning et al. 1974; Wimmers and Turgeon 1991), respectively.

Major attention has recently been given to the development of grass leaves and the distribution of plasmodesmata between their cell types. Virtual absence or paucity of plasmodesmata linking SE/CC complexes with the rest of the leaf symplast were reported from *Zea* (maize) (Evert and Russin 1993; Evert et al. 1996a), *Hordeum* (Evert et al. 1996b) and *Saccharum* (Robinson-Beers and Evert 1991). The plasmodesmal connectivity is, however, decisive for the "pre-phloem transport". In a mutant of maize, abnormal accumulation of starch and lack of phloem export indicated disturbance of phloem loading. Russin et al. (1996) could show ultrastructurally that the plasmodesmata between bundle-sheath and phloem parenchyma cells were covered by wall material and thus discontinuous. Since suberin lamellae in the outer and radial walls of bundle sheath cells form a barrier for apoplastic solutes, at least in maize the assimilate movement into the vein is controlled by the bundle-sheath/phloem parenchyma cell interface (Rusin et al. 1996).



Between different grasses only small differences exist. Grasses have mostly three vein classes and both thin- and thick-walled SEs, with only the former being associated with companion cells. A comparative analysis of  $C_3$  and  $C_4$  grasses in southern Africa showed that the overall plasmodesmal connectivity was correlated with the photosynthetic performance and, accordingly, higher in  $C_4$  plants, thus stressing the importance of intercellular transport between Kranz mesophyll and bundle-sheath cells (Botha 1992). A decrease of the plasmodesmal frequency with increasing proximity to both thin- and thick-walled sieve tubes was found in all four plants studied. A symplasmic route of photoassimilates to the thin-walled sieve tubes, however, could not totally be ruled out in these grasses (Botha 1992).

In grasses, companion cells do not exhibit transfer-cell characters. Therefore, it is noteworthy that Evert and Mierzwa (1989) detected a brush-border-like zone in the inner wall layer of SEs of *Hordeum* that in cross sections appeared as very small, microvilli-like evaginations of the plasma membrane. Besides the obvious enlargement of the plasma membrane surface area, evidence for the role of the brush border is lacking, and the distribution of this feature over other plant families remains to be tested (Evert and Mierzwa 1989).

The role of the thick-walled sieve tubes in grasses is not yet determined. They are connected with pore/plasmodesma contacts to vascular parenchyma cells that neighbour xylem elements. These parenchyma cells were discussed as retrieving sugar from the xylem and transferring it to thick-walled sieve tubes (Fritz et al. 1983). However, according to micro-autoradiography long-distance transport does not seem to take place in thick-walled sieve tubes (Fritz et al. 1989). Plasmodesmata offering an indirect pathway from thick- to thin-walled sieve tubes via the vascular parenchyma and companion cells are again subject to species-specific differences: they are 12 and 63 times more abundant in *Saccharum* and one of the southern African grasses (*Themeda*), respectively, than in maize (Robinson-Beers and Evert 1991).

**Structurally Undefined Phloem Loaders.** Quite a number of plants contrast the clear apoplastic loading type in having several plasmodesmata between bundle-sheath/vascular parenchyma and ordinary companion cells (see "type 2a" of minor veins; Gamalei 1989), even in a plant with transfer-cell-type companion cells (see Fisher 1991). Another combination is given in *Moricandia*, where plasmodesmata crossing the SE/CC complex border are occluded (Beebe and Evert 1992). Apoplastic phloem loading in this species is suggested by the steep concentration gradient between the SE/CC complex and surrounding cells. According to plasmolytic studies, both SEs and companion cells have the same osmotic potential (Beebe and Evert 1992).

Physiological studies are able to delimit the mode of phloem loading. The sensitivity of the sucrose transporters to such sulphydryl-modifying reagents as, e.g., PCMBs mostly served as indicator for apoplasmic loading (Van Bel et al. 1994; Flora and Madore 1996; Ng and Hew 1996). However, even with physiological methods it is very difficult to determine whether all assimilates are loaded apoplasmically at the SE/CC complex border, or whether some assimilates are also arriving at this complex via the symplasmic pathway (in *Ricinus* this "bypass feeding" approaches considerable amounts; Orlich, pers. comm.; see also Orlich and Komor 1992, Komor et al. 1996).

*Molecular Biology of Sucrose Transporters.* Molecular biology seems to offer the chance to unravel how photosynthates get into the phloem of those plants where structural or physiological data are ambiguous (Ap Rees 1994). Only recently, the genes for sucrose transporters have been isolated and characterised from *Spinacia* (spinach), *Solanum* (potato) (Riesmeier et al. 1992, 1993), *Arabidopsis*, *Plantago* (Gahrtz et al. 1994; Sauer and Stolz 1994) and *Ricinus* (Weig and Komor 1996). The decisive step for the first isolation of a sucrose transporter cDNA by Riesmeier et al. (1992) was the complementation cloning in a yeast strain that due to a gene deletion lacked both an endogenous sucrose transporter and invertase secretion, and was therefore unable to grow on a sucrose medium. After transformation of the yeast strain with a cytosolic sucrose synthase or invertase and a cDNA expression library from spinach, the selection occurred on a medium with sucrose as the only carbon source. Thus, yeast transformants with a full-length plant sucrose transporter gene could be identified and the protein characterised (Riesmeier et al. 1992). A high degree of sequence homology between the different species and a similar structure with two times six membrane-spanning domains denote the protein to a super family of higher plant membrane transporters (Sauer and Tanner 1993).

Different approaches were undergone to test the phloem specificity of the transporters. In situ hybridisation showed a high expression level of the sucrose transporters in the phloem of potato source leaves (Riesmeier et al. 1993). The promoter of the *Arabidopsis* sucrose transporter directed the  $\beta$ -glucuronidase (GUS)-expression equally to the phloem (Truernit and Sauer 1995). The cDNA of the *Plantago* transporter was cloned by screening the cDNA library from isolated vascular bundles with the DNA of the *Arabidopsis* transporter (Gahrtz et al. 1994). Isolation of the bundles reduced the cDNA library to vascular genes, most of which should originate from living phloem tissue.

Antisense constructs of the transporter showed an inhibition of the photosynthate export from mature leaves in *Solanum* transformants. This resulted in the accumulation of hexoses, sucrose and starch within source leaves (Riesmeier et al. 1994). A companion-cell-specific an-

tisense inhibition by the *rolC*-promoter also led to the accumulation of sugars and starch in the leaf. The phenotypic effects were light-dependent (Kühn et al. 1996). In contrast to tobacco plants overexpressing yeast invertase (Ding et al. 1993), the development of plasmodesmata was arrested in neither green nor yellow sectors of source leaves from antisense plants with a strongly reduced transporter mRNA level (Kühn et al. 1996).

The antisense transformants give direct evidence that in *Solanum*, phloem loading at least partly depends upon the sucrose transporters. Accordingly, the bottleneck in the export of assimilates is the transporter activity. The presence of quite a number of plasmodesmata in all interfaces between mesophyll, bundle-sheath, phloem parenchyma and companion cells (McCauley and Evert 1989; for other species see Gamalei 1989) might form a symplasmic bypass pathway that – according to the antisense transformants – however, does not suffice for all assimilates.

*Immunocytochemical Localisation of Sucrose Transporters.* Final evidence for the role of the isolated sucrose transporters was achieved by their localisation. The transporter protein was localised by immunofluorescence to the companion cells of *Plantago* and *Arabidopsis* (Stadler et al. 1995, Stadler and Sauer 1996). The identity of the cell type was determined by positive DAPI fluorescence (SEs are negative) and by the typical side-to-side arrangement of the companion cells with the SEs, the latter being identified by their callose fluorescence at sieve plates. This localisation fitted well with the preferential localisation of the  $H^+$  ATPase to the plasma membrane under wall ingrowths in *Vicia* (Bouché-Pillon et al. 1994) and localisation to *Arabidopsis* companion cells of the phloem-specific AHA3-ATPase (DeWitt and Sussman 1995).

It was, however, a surprise when in mature *Solanum* phloem the sucrose transporter protein appeared not in companion cells but in the SEs of leaf and petiole phloem according to immunofluorescence microscopy (Kühn et al. 1997). EM-immunogoldlabelling confirmed the localisation in *Solanum* and *Nicotiana* and depicted the transporter at the SE plasma membrane of leaf, petiole and root metaphloem. Moreover, in situ hybridisation at the EM-level showed the mRNA of the transporter in both companion cells and SEs (Kühn et al. 1997). The mRNA and protein levels are light-dependent and have high turnover rates. Already nucleate SEs show immunofluorescence of the transporter at their plasma membrane (Kühn et al. 1997). These results indicate that the sucrose transporter is one of the first specific characters in SE differentiation. Furthermore, in mature SE/CC complexes its turnover is regulated and programmed in companion cells. The pore/plasmodesma contacts are obviously involved in the transfer of the protein and/or the mRNA into the SE. The significance of the considerable amount of transporter

mRNA in enucleate SEs remains unclear. A translation of the protein is inconceivable in cells without ribosomes. Further studies are needed to delimit the problem and exclude both possibilities: that the mRNA is artificially dragged from the companion cell into the SE, and that the mRNA just belongs to the leftovers of the nucleate SE stages. If this can be excluded, the intercellular movement of nucleic acids and signal proteins is obviously relevant to the present discussion which involves this movement with the short- and long-distance coordination of plant cells (see Lucas 1995; Lucas et al. 1996). With regard to the driving force of the active uptake of sucrose in Solanaceae, the next step is to solve whether the ATPase co-localises with the transporter in the SE or is active in the companion cell (Van Bel 1996b; Kühn et al. 1997).

The discrepancy in the immunolocalisation of the sucrose transporters exemplifies that – also for the apoplasmic mode of phloem loading – different plant families have developed a different division of labour among the phloem cell types. This applies also to other proteins involved in sucrose uptake: an antibody against a sucrose-binding membrane protein labelled the *companion cell* in mature soybean phloem (Grimes et al. 1992) and the plasma membrane of SEs in mature spinach phloem (Warmbrodt et al. 1989). This sucrose-binding protein was discussed as mediating the linear component of sucrose uptake in a non-saturable manner and as being independent of and different from the active, saturable co-transport of sucrose shown by ordinary sugar membrane transporters (Overvoorde et al. 1996, cf. Sauer and Tanner 1993).

It is obvious that due to the new analytical approaches phloem loading has again become a prosperous topic for plant scientists. Taken together, classical physiology, molecular and structural cell biology can be expected to solve most problems of the first and key step in the long-distance transport of assimilates in the near future. The further steps in translocation, i.e. the partitioning of assimilates between two (or more) sinks and unloading, can be described by a simple mechanistic model of phloem transport rather than by an active and highly regulated mechanism (Minchin et al. 1993).

## b) Transport Phloem

The physiology of the transport phloem was treated only recently in *Progress in Botany* (Van Bel 1993b). For the transport function the relative symplasmic discontinuity with the surrounding tissue appears significant. It is among others expressed by steep differences in the membrane potential (Van Bel 1993b). The transport phloem is to a certain extent able to take up and release translocates. Release may be considered as leakage or as lateral unloading. It was assumed that the active uptake serves as a retrieval mechanism for keeping the translocates within the conduit (Grimm et al. 1990; Van Bel 1993b and literature cited

therein). Though there was no doubt that both processes – the lateral loss and lateral loading – of the transport phloem occur, a recent publication using C-11 as transport marker and PCMBS and CCCP (carbonyl-cyanide-*m*-chlorophenyl-hydrazone) as inhibitors indicate that phloem transport is not influenced by a blockage of lateral loading (Grimm et al. 1997). According to the authors, it has to be clarified how lateral sealing of the translocation path is influenced by the (terminal) sink strength, permeability of the sieve-tube membrane and the speed of translocation (Grimm et al. 1997).

The phloem elements show few specific structural adaptations to the transport function. Generally, the basic structure of SEs, as described in Section 3, seems already optimised for a low-resistant conduit. Any further specialisation for transport essentially concerns the width of the sieve pores. In the secondary phloem of angiosperm trees the sieve pores reach the largest diameters: 7- $\mu$ m pores were documented in *Fagus* (by EM; Schulz and Behnke 1986) and 15- $\mu$ m pores in *Ailanthus* (by LM; Esau and Cheadle 1959).

#### $\alpha$ ) Wound Response

Widening of the pores to the above values means an increased risk of wound-induced losses of translocates. Accordingly, wound responses have developed that include the constriction of the pore channel, the plugging of the pore channel, both in angiosperms (see Schulz 1986b; Galway and McCully 1987), and the swelling of the ER tubules covering the sieve areas in gymnosperms (Schulz 1992).

Plugging of pores by cytoplasmic contents and constriction of the pore channel by callose production are apparently evoked by the sudden pressure release when a sieve tube is cut. The pressure release might also induce entry of calcium from the apoplast, in so far as mechanosensitive ion channels at the sieve pores are effective (see Pickard and Minchin 1990, 1992b). Changes in the permeability of the plasma membrane allowing entry of apoplasmic calcium and its compensation by potassium were discussed as activating callose synthase, localised in the plasma membrane (see Kauss 1987; Delmer et al. 1993). Phloem exudation was often experimentally facilitated by application of calcium chelators (e.g. King and Zeevaert 1974; Urquhart and Joy 1981, Van Bel et al. 1994). A comparative functional and structural study on the effects of calcium and chelators on phloem exudation from cut pea root tips revealed that callose production is a secondary wound response (Schulz 1996a; Schulz and Pancke 1996a,b). Exogenous calcium effected also a crosslinking of accumulated P-protein filaments so that translocation of radiolabelled assimilates came to a sudden stop. This response is in a way comparable

with the well-known gelling of the P-proteins in cucurbits after their exposure to air (see Cronshaw and Sabnis 1990).

Still another response to wounding was detected in gymnosperm sieve areas close to purposeful cuts. The ER tubules of the complexes covering the sieve areas swell on the wound-exposed side, but not on the opposite side. It was concluded that in intact sieve cells the tubular ER is in equilibrium with the sugar concentration of the SE lumen. Accordingly, the pressure release after wounding leads to water endosmosis into the ER and thus to the swelling of the tubule up to the point where these plug the sieve pores efficiently (Schulz 1992).

### β) Phloem Transport in Gymnosperms

The effective sieve-area blockage elucidates the problem arising from the complex composition of sieve-area pores and the role of the ER in gymnosperms compared with the simple pores of angiosperms. In addition, the latter are wider and much less occluded by cytoplasmic material (Sect. 3.d.β). After studying the conifer phloem *in vivo* with a confocal laser scanning microscope, Schulz (1992) discussed an active participation of the ER complexes in phloem transport. Enzyme localisation, both at the LM and EM levels, showed nucleoside triphosphatase and glycerophosphatase at the ER complexes (Sauter 1976, 1977). An active role of the ER is also supported by its staining with the cationic dye DiOC (3,3'-dioxacarbocyanine iodide). This dye is supposed to mark membranes that have a significant membrane potential, with the negative charge inside (Matzke and Matzke 1986). The activity of nucleoside triphosphatases at the ER complexes, a proton gradient across these membranes and, concomitantly, a high membrane surface were taken as indication that phloem transport in gymnosperms does not only depend on loading in source leaves and unloading in sinks, but requires also energy consumptive steps within the path (Schulz 1992).

The ER forms a non-plasmatic compartment that is continuous through sieve areas. It was suggested that this compartment is able to regulate the long-distance gradient of assimilates by reestablishing or steepening the gradient in each sieve cell (Schulz 1992). This hypothesis is supported by accumulation of C-14 translocates on the sieve areas in micro-autoradiographs (Schmitz and Schneider 1989), by the fact that gymnosperm sieve cells might approach a length of 5 mm (Esau 1969) and by the size records held by the *Sequoia sempervirens* (Californian redwood) trees (see Schulz 1990a). Here, the secondary phloem provides a more than 100-m-long transport conduit between the green needles and roots of *S. sempervirens*. It is hard to believe that a gradual sugar gradient over this length, only built up by phloem loading in needles, allows for an effective translocation within the phloem (see Schulz 1992

for the relationship of this proposal to the relay hypothesis of phloem transport; Murphy and Aikman 1989).

#### γ) Lateral Sinks

Transport phloem is capable of taking up and releasing assimilates. Unloading is important in secondary phloem of trees where the lateral transfer of assimilates via rays cells leads to starch storage and supplies the growth of wood and bark increments (Sauter and Kloth 1986; see also Van Bel 1990). Artificial lateral sinks are aphids that suck functional SEs and live upon their amino acids. Besides these insects also higher plant parasites abuse the phloem system of their hosts (for a review see Dörr 1990). The holoparasites act as dominant sink and retract a large proportion of assimilates, thus exhausting the host. Different parasite species show different modes of lateral assimilate uptake. Specialised absorbing haustoria may or may not come into direct contact with the host phloem (Dörr 1990). *Cuscuta* is a parasite that is most specialised in an apoplasmic transfer of assimilates from host sieve tubes to haustorial cells which later differentiate into SEs. The contact zone between absorbing hypha and host sieve tubes has transfer-cell character (Dörr 1990). This transfer does not seem to involve invertase activity (Neumann and Haupt 1996). In contrast to the apoplasmic mode of assimilate removal by *Cuscuta*, primary sinkers of the root parasite *Orobancha* achieve direct symplasmic contact with the host phloem. Sieve pores between host and parasites, each identified by specific cell marker, have the well-known ultrastructure (Dörr and Kollmann 1995).

#### c) Release Phloem

Phloem unloading presumably occurs to a certain extent all along the conduit (see Sect. 4.b) but prevails in the target sink tissues. Evidence has accumulated that in most instances phloem unloading in the narrow sense (i.e. SE unloading) takes place symplasmically (Patrick 1990, 1997). Exceptions from this rule were derived from the absence of plasmodesmata or a lack of movement of fluorescent markers across the SE/CC complex boundary and were found in maize leaves (Evert and Russin 1993), several fruits and sucrose-storing tissues. For a tabular survey of the documented cases of sink unloading see Fisher and Oparka (1996).

### $\alpha$ ) Symplasmic Unloading

The overall picture of a symplasmic mode of unloading from SEs fits nicely with the generalisation that unloading is down-hill, i.e. along the turgor gradient. Apoplastic unloading from the SE/CC complex would involve two, thermodynamically doubtful, membrane steps: the release of sucrose into the apoplast and, from there, the active uptake of sucrose (or, when apoplastic invertase participates, of hexoses) by parenchyma cells. The occurrence of plasmodesmata, the movement of fluorescent and radioactive markers and plasmolytic studies support symplasmic unloading from protophloem in root apices (Dick and Ap Rees 1975; Warmbrodt 1985, 1987; Oparka et al. 1994; Schulz 1994), from the phloem of developing cereal and legume grains (Wang and Fisher 1994a,b; Fisher and Wang 1995; Patrick and Offler 1995), potato tubers (Oparka 1990) and developing leaves (Ding et al. 1988). As was pointed out by Van Bel and Oparka (1995) the existence of plasmodesmata, however, can only show the option for symplasmic transport and must be substantiated physiologically.

Even if this is done, the drawbacks of most markers for symplasmic transport as, e.g., their pH sensitivity, the uncertain substructure of plasmodesmata and problems arising from non-specific effects by inhibitors like PCMBs (see Tucker et al. 1989; Ding et al. 1992; Dorhout and Kollöffel 1992, Wright and Oparka 1996) may cast doubts on the validity of symplasmic unloading, even in the generally agreed case of unloading from protophloem-sieve tubes in roots. Thus, theoretical considerations and calculations of the sucrose fluxes in maize roots led Bret-Harte and Silk (1994) to conclude that the demand of the growing root tip can not be met by the symplasmic diffusion of sucrose from the phloem.

Provoked by this approach Schulz (1996a,b) tested the opposite hypothesis, i.e. whether apoplastic rather than symplasmic unloading is plausible from protophloem-SEs. Pea seedlings were used and the dry weight of root segments, as well as the number, length and diameter of their protophloem- and metaphloem-sieve tubes, were determined from serial EM cross sections. Notably, protophloem-sieve tubes of pea roots are without companion cells (Schulz 1995). With these data, the net carbon import per segment and the membrane surface of the SEs in each segment could be calculated. According to this unloading analysis, fluxes of sucrose equivalents across the SE boundary amounted to nearly  $700 \text{ pmol} \cdot \text{cm}^{-2} \cdot \text{s}^{-1}$  in the apical segment (Table 1, line 15: "A+B"). This value is 20–50 times higher than any known membrane fluxes (for active loading into transfer cells,  $14 \text{ pmol}$ ; see Gunning et al. 1974 and Sect.4.a.β), although respiration, amounting to 42% of imported carbon (Farrar 1985), was not accounted for. The extreme high unloading rate of



Table 1. Sieve-element unloading in the root tip of pea seedlings cannot occur apoplasmically, since the unloading rate is 20–50 times higher than the upper limits of known transmembrane fluxes (Schulz 1996a)

Measurements		Segments A B C D E F G H										
Weights	FW	(1)	mg	3.220	4.025	4.448	4.538	4.568	4.888	5.025	5.115	
	DW	(2)	MG	0.515	0.358	0.310	0.328	0.363	0.393	0.428	0.430	
	H <sub>2</sub> O	(3)	μl	2.705	3.668	4.138	4.210	4.205	4.495	4.598	4.685	
		(4)	N	18	35	49	63	77	90			
Sieve tubes (ST)	Basal Ø area	(5)	μm <sup>2</sup>	712	1385	1939	2493	3047	3561			
	Length	(6)	mm	57	132.5	210	280	350	417.5			
	Surface	(7)	mm <sup>2</sup>	1.43	3.33	5.28	7.05	8.81	10.51			
	Volume	(8)	nl	2.26	5.24	8.31	11.08	13.85	16.52			
Calculations		Apical segments A+B A-D A-F										
Root tip import	SMT	(9)			0.978	1.158	1.315			mg DW d <sup>-1</sup>		
		(10)			2.9	1.9	1.5			g cm <sup>-2</sup> h <sup>-1</sup>		
	Sucrose equiv.	(11)			1.98	2.35	2.67			nmol min <sup>-1</sup>		
	Axial flux of sucrose	(12)			2.39 x 10 <sup>6</sup>	1.57 x 10 <sup>6</sup>	1.25 x 10 <sup>6</sup>			pmol cm <sup>-2</sup> s <sup>-1</sup>		
Import per 10-mm segment			10-mm segments A+B C+D E+F									
	Gain	(13)			0.978	0.180	0.157			mg DW·d <sup>-1</sup>		
	Sucrose equiv.	(14)			1.98	0.37	0.32			nmol min <sup>-1</sup>		
	Unloading of sucrose	(15)			693.47	49.43	27.53			pmol cm <sup>-2</sup> s <sup>-1</sup>		

For calculation of dry matter demand in a growing root segment, the demand apical of a border between segments was the final dry weight (DW) minus the starting weight, transformed into DW increase per day (9); cf. Bret-Harte and Silk (1994). Calculations were to 10-mm segments in order to include the entire growing zone of the apex. For measurements, fresh and dry weights of 5-mm segments ( $n = 40$ ), starting with the apical one (A), were recorded. Sieve-tube numbers (4) are from cross sections at basal end of each segment (*italicised: interpolated numbers*). Growth rate of roots (15.34 mm day<sup>-1</sup>;  $n = 40$ ) is from Schulz (1994). FW, fresh weight; DW, dry weight; SMT, specific mass transfer.

protophloem-sieve tubes excludes a substantial participation of apoplastic unloading in the first 10 mm of pea roots (Schulz 1996a,b).

In older root segments, the phloem consists of proto- and metaphloem-sieve tubes, the latter of which have companion cells. Here, the unloading rate is much smaller (about  $30\text{--}50 \text{ pmol} \cdot \text{cm}^{-2} \cdot \text{s}^{-1}$ ; Table 1: line 15: "C+D"; "E+F"), since it supplies maintenance metabolism only. Being close to the limits of membrane transport, apoplastic unloading is more realistic from the metaphloem than from the protophloem. The plasma membrane of the companion cells of metaphloem-sieve tubes may contribute to this process. Participation of symplasmic unloading – suggested by the existence of plasmodesmata in all wall interfaces (Warmbrodt 1985; Schulz, unpubl.) – may add to the unloading balance.

In conclusion, the calculation of the unloading rate of sieve tubes by classical physiological methods and morphometric analysis of EM micrographs is a valuable means to determine the major mode of unloading. The result of symplasmic unloading in pea roots does not necessarily contradict the basic theoretical results of Bret-Harte and Silk (1994), though for pea roots a few assumptions of the authors should be corrected (see Schulz 1995). The authors discussed that their theoretical results indicate either an incomplete understanding of the plasmodesmal structure and function or the existence of an alternative sucrose transport mechanism for the transport of sucrose towards the meristem. The former appears more likely because plasmodesmata were shown to generally have an extended SEL in unloading tissue (see Wang and Fisher 1994a,b; Fisher and Wang 1995, Patrick and Offler 1995) or to be able to widen according to physiological demands.

### β) Changes in Symplasmic Phloem Unloading by Changes in Plasmodesmal Conductivity

The symplasmic phloem unloading rate in pea roots could be experimentally increased by the application of mild osmotic stress to the root tip (Schulz 1994). The most affected expanding cortex cells coped with the imminent water exosmosis by the attraction of assimilates from the protophloem. Similarly treated root tips were subjected to high-resolution EM and the substructure data of the plasmodesmata along the unloading pathway were recorded (Schulz 1995). Compared with controls, a transient, highly significant increase in the passage area of plasmodesmata was found in root tips treated for 1 h with the osmoticum mannitol. The increase was due to the widening of the neck constriction, while the diameter of the desmotubulus remained unchanged (Schulz 1995). Structural and functional regulation of the plasmodesmal passage were correlated: per 100 plasmodesmata the passage area as well as the

unloading rate were roughly three times the control values (Schulz 1995). The release of the plasmodesmal neck constriction could be due to the involvement of cytoskeletal elements. These might actively (under ATP control) keep plasmodesmata constricted and may be released whenever assimilates are needed (see also Cleland et al. 1994; White et al. 1994; Ding et al. 1996). Other mechanisms, however, such as, e.g., callose synthesis and removal or the conformational changes of desmotubular proteins might as well participate in the regulation of the plasmodesmal passage area (Lucas et al. 1993; Lucas 1995).

The correlation of the unloading rate of labelled assimilates with the plasmodesmal widening in the root cortex (Schulz 1994, 1995) indicates that the entire symplasmic pathway from the SEs to the receiver cells behaves as an extension of the phloem domain so that changes in demand within the expanding cortex parenchyma evoke an immediate feedback to the assimilate gradient in the phloem conduit.

#### γ) Apoplastic Unloading

From the structural point of view, there does not seem to be a specialisation of phloem elements for unloading. Even in cases where an apoplastic unloading is suggested by the absence of plasmodesmata at the SE/CC border, structural adaptations of the cells involved are lacking (Evert and Russin 1993). To the best of the author's knowledge, transfer cells are not formed in release phloem, though they might be very important later in the post-phloem transport, e.g. at the maternal/filial interface (Patrick and Offler 1995).

While "unloading" along the phloem conduit might be considered as leakage except for lateral sinks, a different case are sucrose-storing tap roots of sugar beet. Here, an apoplastic mode of unloading was discussed, since unloading towards the sugar-storing cells implies an up-hill transport from the phloem (see Patrick 1990, 1997). A final decision about the mode of unloading in sugar beet roots, however, appears immature unless the role of plasmodesmata and the compartmental balance kept by the membrane transporters are understood. In a preliminary report only a few plasmodesmata were described as occurring between the secondary phloem and the storage parenchyma cells (Mierzwa and Evert 1984) which derive from anomalous cambia in beet roots. Sucrose transporters occur in both plasma membrane and tonoplast of the storage parenchyma cells, and show a symporter and an antiporter character for protons, respectively (see Fieuw and Willenbrink 1990; Getz and Klein 1995a,b).

## 5. Conclusions

The SE structure is optimised for assimilate transport in offering a low-resistance pathway. Intracellularly, it is introduced by the selective autophagy of the SE protoplast realised in all plant groups. With increasing longevity, however, the enucleate SEs become increasingly dependent upon associated cells, so that in angiosperms the association is ontogenetically guaranteed. Intercellularly, the low-resistance pathway is established by specialised plasmodesmata, the sieve pores. Plasmodesma specialisation involves an extreme widening of the cytoplasmic sleeve to a pore (by up to 200 times in diameter) and the abandoning of an obligatory ER component in all vascular plants except for gymnosperms and some fern species.

Among the persistent entities in mature SEs, the role of the plastids and their inclusions, of the SER and of the soluble and structural phloem proteins deserve further research efforts. Speculations of their commitment in loading, retrieval, protein turnover and wound response have to be tested with physiological, structural and molecular biological methods. In particular, this applies to the movement of proteins between SE and companion cells and across graft unions, since it has far-reaching consequences for the understanding of phloem transport.

Considering the structural adaptation of the phloem to its functional tasks – collection, transport and release of assimilates – it is obvious that the structural prerequisites for transport and release of assimilates are already provided by the "normal equipment" of the SE and its associated cell. Specific adaptations to the functional task are only evident from the collection phloem in source leaves.

Evidence is overwhelming to date that more than one mode of loading is realised in different plants. The symplasmic mode is structurally indicated by a high plasmodesmal connectivity of the companion or Strasburger cells with the leaf symplast via branched or otherwise specialised plasmodesmata. A combination of sucrose and tri- or tetrasaccharides or polyols as transport sugar adds evidence to a symplasmic mode of loading.

The apoplasmic mode is well established and confirmed by physiological and molecular biological investigations. Structurally, this mode of loading is suggested wherever the SEs are symplasmically isolated and/or where the associated cells have transfer-cell character. The division of labour among the SE and associated cell is obviously diverse in different apoplasmic loaders as indicated by the variability of the location of the respective enzymes and transporters. Evidence is also accumulating that plants may have the potential for mixed modes of phloem loading that might occur parallel or alternative, depending on the growth condition.

The in-vivo structure of the sieve areas of gymnosperms appears inconsistent with a simple Münch-type phloem transport mechanism. It was postulated that the ER complexes covering the sieve areas are actively involved in both phloem loading and transport in gymnosperms. Guided by the specific ultrastructure, Kollmann and Schumacher discussed in 1964 that the sieve cell ER might be involved in the transport function. In the belief that particular structures – if reconciled by different methods – reflect specific functions, the author wishes today to renew the call for physiological investigations into the long-distance transport of assimilates in gymnosperms.

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# The Mystery of Virus Trafficking Into, Through and Out of Vascular Tissue

By Richard S. Nelson and Aart J. E. van Bel

## 1. Introduction

A simple way to look at the process of plant virus infection is as a board or computer game. The objective for the virus is to move from the site of inoculation throughout the rest of the host plant, replicating and accumulating at specific areas along the way. The host plant serves as the game board. To "win" this game, a virus must find the most efficient way to infect the entire plant. Since viruses encode only a portion of the proteins necessary for this challenge, they must enlist (i.e. usurp) plant host factors to aid them in this journey. They also must avoid defence mechanisms employed by the host to limit their systemic spread.

Not one aspect of this journey is understood in complete detail. Our understanding of the mechanisms for each process in infection ranges; it is modest for *in vivo* virus assembly or disassembly (e.g. Heaton and Morris 1992; Register and Nelson 1992; Verduin 1992; Fox et al. 1994), moderate for virus cell-to-cell movement (e.g. Citovsky and Zambryski 1993; Lucas and Gilbertson 1994; Sanderfoot and Lazarowitz 1996) and considerable for virus replication (e.g. Covey and Hull 1992; Lazarowitz 1992; Buck 1996). Even in the case of virus replication, however, much is left to determine [e.g. how the viral components of the replicase for RNA viruses interact to initiate RNA synthesis (Buck 1996) and with which host factors they associate (Hayes and Buck 1990; Quadt et al. 1993)]. Our understanding of host resistance mechanisms is also limited as only one host resistance factor has been identified (Whitham et al. 1994).

The process of vascular-dependent infection by viruses is another area where our knowledge is incomplete, even though scientists have suspected for 60 years that many plant viruses rapidly invade distant tissue through the vasculature (Holmes 1930; Samuel 1934; Bennett 1940). Much of the difficulty in obtaining information on this process was due to inadequate technology. The vasculature is embedded within the plant tissue and thus not readily observable through microscopy without damaging the tissue. Damage of vascular tissue either during tissue sampling or fixation usually leads to significant and sometimes irreversible alterations in vascular tissue structure (see comments by Overall



and Blackman 1996 regarding effects of wounding and fixation on plant tissue). These alterations can be rapid and inevitably raised questions about potential artifacts appearing in the observed tissue. This notwithstanding, the analysis of virus spread through vascular tissue has seen some major leaps forward. The first occurred in the 1960s and involved the use of the electron microscope to study the accumulation of virus in vascular tissue. Virus particles were seen in particular cell types and thus the ability of virus to accumulate in cells around and within the vascular tissue was defined to the limits of that technology. Later, with the availability of highly specific antibodies against proteins expressed from cloned viral genes and cDNA probes complementary to viral genes, extremely specific immunocytochemical and *in situ* hybridization studies have been conducted. Results from these studies have helped to further identify the cell types in which virus is present and to solidify the contention that virus is replicating in these cells and not simply accumulating upon transport.

Recently, the ability to express marker proteins [e.g.  $\beta$ -glucuronidase (GUS), luciferase, green fluorescent protein (GFP)] either in a free form or as a fusion with a viral protein from infectious transcripts of cloned viruses or transient expression vectors has given researchers a powerful tool to follow virus or viral protein movement into, through and out the vasculature. Inoculation or injection, respectively, of "labeled" viruses or viral proteins into specific cells will allow for the assessment of virus or viral protein movement between cells and their association and effect on plasmodesmata (PD) spanning these cells. Confocal laser microscopy now enables researchers to observe virus infections deeper into live intact tissue. Software advances (e.g. three-dimensional digital analysis packages) in combination with conventional microscopy may give a greater number of researchers the ability to obtain results similar to those obtained with the confocal microscope for less capital outlay (Carrington et al. 1995; Casavan et al. 1996).

The increasing availability and use of these technical advances to study the vascular movement of viruses is now accelerating the rate at which we are demystifying this process. Basic questions which previously were unaddressable can now be addressed. For example, we can now determine whether or not plant viruses move through the same cells that photoassimilate passes through during phloem loading. We will also be able to determine the form in which plant viruses move through the vascular tissue. As a final example, we should now be able to identify the veins utilized for viral exit into sink leaves and the path of infection from those sieve elements in the sink leaves to other cells.

In this chapter, we will first lay out the game board; that is, give a general description of the host vein anatomy encountered by the virus in source, transit and leaf sink tissue. A description of the known functions for the particular cells within the veins will be included. We will then

present the known information about the movement of viruses along the pathway, organizing each section to discuss movement between specific cell types. It is likely that further understanding of virus trafficking in vascular tissue will also increase our understanding of trafficking mechanisms for host-encoded proteins and photoassimilates. Potential areas of overlap between host protein or photoassimilate trafficking and virus trafficking will be highlighted. Within sections we will raise questions that currently are unanswered and suggest how the new technologies may aid in answering these questions.

Many reviews on plant vascular and plasmodesmal anatomy and physiology (e.g. van Bel 1993; Lucas 1995; van Bel and Oparka 1995; Grusak et al. 1996; Overall and Blackman 1996; Turgeon 1996; Ding 1997; van Bel and Kempers 1997) and cell-to-cell and, to the extent allowed by the research, vascular-mediated virus movement (e.g. Dawson and Hilf 1992; Deom et al. 1992; Gilbertson and Lucas 1996; Leisner and Turgeon 1993; Lucas and Wolf 1993; Lucas and Gilbertson 1994; de Zoeten 1995; Carrington et al. 1996; Oparka et al. 1996; Séron and Haenni 1996) have appeared recently and the reader should utilize these articles for further information on this topic.

## **2. Synopsis of Minor and Major Vein Ontogeny and Function in Mature and Immature Leaves**

### **a) Anatomy and Functional Domains of the Phloem and Xylem Systems**

The phloem system serves to distribute photosynthate over long distances and, hence, is vital for the development and concerted growth of higher plants. The phloem is subdivided into three functional units each having a specific task (see van Bel and Kempers 1997). At the beginning of the system, the phloem collects the photosynthate, while at the end of the system the photosynthate is released into the centres of growth or the storage organs. The intermediate transport phloem executes the translocation of photosynthate between the collection and release zones. Functionally, the transport phloem is a hybrid between the collection and release phloem in view of the balanced release/retrieval of photoassimilates along the pathway (Minchin and Thorpe 1987). The decreasing energy requirements for photosynthate retention sequentially in the collection, transport and release phloem is positively correlated with the decreasing volume ratio between companion cells (CCs) and sieve elements (SEs) along the phloem pathway. Whilst the CCs are much larger than the SEs in the phloem loading zone, the CCs cover only about 30% of the SE-surface in the transport phloem of highly developed dicotyledons, and are again smaller in the phloem unloading zone (van Bel and Kempers 1997).

The use of the term "zone" in "loading zone" and "unloading zone" has a significant meaning; photosynthate transport by the phloem is strongly linked with carbohydrate processing in the pre-SE and post-SE pathways (trajectories). The general opinion is that most viruses employ the phloem system, including the above-mentioned short-distance trajectories most likely to the minor veins, for their rapid spread through the plant (e.g. Leisner and Turgeon 1993; Gilbertson and Lucas 1996). This implies that viruses must access a pathway structurally and functionally designed for photosynthate transport and, hence, must adapt to the limitations of this system.

Additional requirements must be fulfilled by viruses moving in the xylem system. They must be equipped with tools to cross membrane barriers and withstand intercellular (i.e. apoplasmic) conditions, since virus multiplication takes place within the symplasm and xylem translocation of virus is restricted to apoplasmic compartments. For solute movement in xylem, the pathway of entry into the system appears more uniform than that for phloem. There is a symplasmic continuum extending from, at the minimum, the cortex to the xylem parenchyma in primary roots (Warmbrodt 1985a,b, 1986). Hence, solutes can be transported from the cortex through pericycle and endodermis to the xylem parenchyma cells. From the xylem parenchyma cells the solutes are likely actively released into the xylem vessels (Wegner 1996; de Boer and Wegner 1997). Theoretically, viruses may also follow this pathway with the implicit difficulties of membrane permeation. Another potential pathway for invading the xylem system is that the viruses spread symplasmically from the sink phloem into the root meristem cells (Chambers and Francki 1996; Dubois et al. 1994; also see Sects. 2.e, 5.a). When the precursors of the vessel members die off at maturity, the viruses are massively released into the xylem system. The latter scenario for virus movement into xylem might also be envisioned to occur in the shoot apex. In all cases, exit from xylem remains a mystery.

The osmotic potential built up in the root xylem produces, together with the transpiration, the driving force for mass flow through the xylem. Along the xylem pathway the chance for the viruses to escape via the virtually water-impermeable (Wisniewski et al. 1987), lignified walls seems small. Alternatively, the radially oriented intercellulars between the ray cells may offer a quick lateral apoplasmic passageway for the viruses (reviewed by van Bel 1990). Further, the bordered pits may provide a way out as the virus may be able to move through the primary cell wall material of the xylem parenchyma cells adjacent to the pits (reviewed by van Bel 1990). The xylem parenchyma cells, often strategically situated around the vessels, exert a tight control on the solute composition in the vessels. The parenchyma collar around the vessels is composed of a longitudinal domain and/or radially oriented symplast domains (van der Schoot and van Bel 1990). Within leaves, the route and

mechanism of water movement in the minor veins and vein endings is unclear (Evert et al. 1985; Eastman et al. 1988b; Canny 1990).

The next section will focus on the structural and physiological difficulties viruses encounter in movement within the collection, transport and release zones of the phloem. In the collection zone, virus would be assumed to move through plasmodesmata (PD) until it comes to the bundle sheath cell (BSC)/vascular parenchyma cell (VPC) interface with the CCs. At this point the route of virus movement into the CCs is unclear, as, in many plant species, photoassimilates move through the apoplast and viruses likely stay in the symplast at this junction. For these plants, viruses would clearly have to modify this system or find a symplasmic route to the SEs. In the long-distance transport zone, the physiological environment in the sieve tubes might be far from ideal for virus survival. In the release zone, structural and physiological limitations again are likely. Limited attention will be devoted to the difficulties that xylem-transported viruses experience, as knowledge about these viruses is scanty.

## b) Functional Domains of the Phloem Loading Zone

### $\alpha$ ) *Dicotyledons*

In dicotyledons, the vein system is mostly reticulate (Hickey 1979). From a midvein (lowest-order or first-order vein), vein orders of reducing complexity diverge. In ancient dicotyledonous families, the number of vein orders is mostly limited to three or four. In leaves of modern dicotyledons, the number of vein orders can increase to seven. The highest-order veins (usually fourth order and above) are considered minor veins, are embedded in the mesophyll and are the principal, if not the only, sites of photosynthate collection and initial export (Komor 1977; Turgeon and Wimmers 1988; Turgeon 1989; Gamalei 1990; Figs. 1, 2). Given the paradigm that viruses follow the photosynthate pathway (see Sects. 3b, 6), the anatomy of these veins and the adjoining non-vascular elements is discussed below.

In the pre-SE trajectory, photoassimilates sequentially move from the mesophyll cells (MCs) through one or occasionally two layers of parenchymatous BSCs to the VPCs or directly to the SE/CC complexes. The number and structure of cells in the trajectory are respectively variable and complex. In numerous species, plasmodesmal densities between the extravascular elements have been documented (Russell and Evert 1985; Evert and Mierzwa 1986; Fisher 1986; McCauley and Evert 1989; Bourquin et al. 1990; Warmbrodt and Van der Woude 1990; Beebe and Evert 1992). From these studies a few trends emerge. The plasmodesmal density between palisade parenchyma cells is often lower than that between

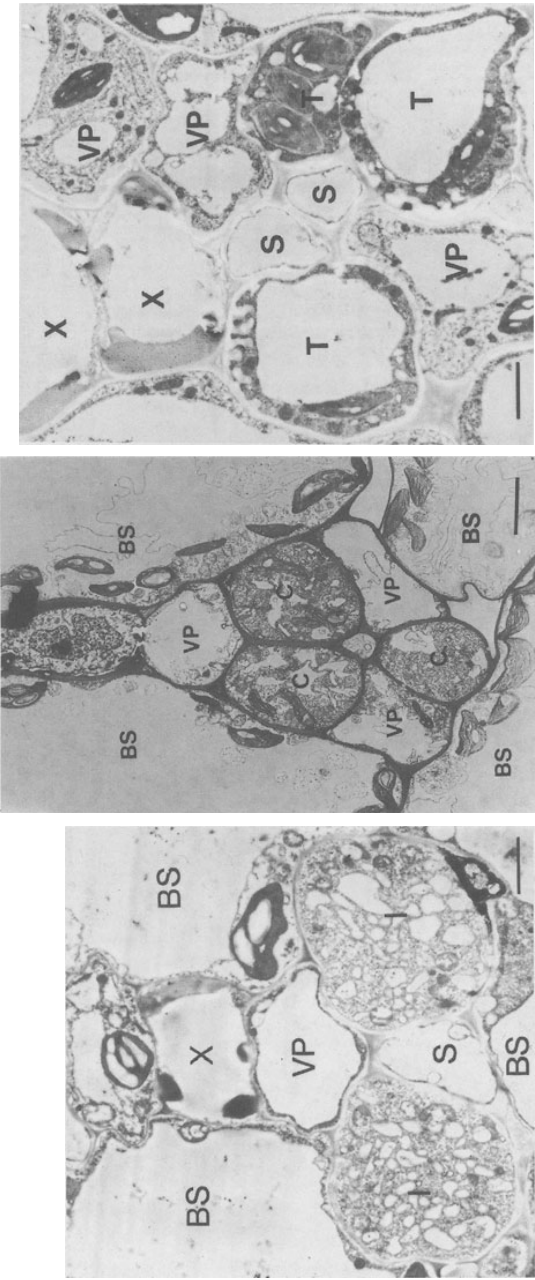
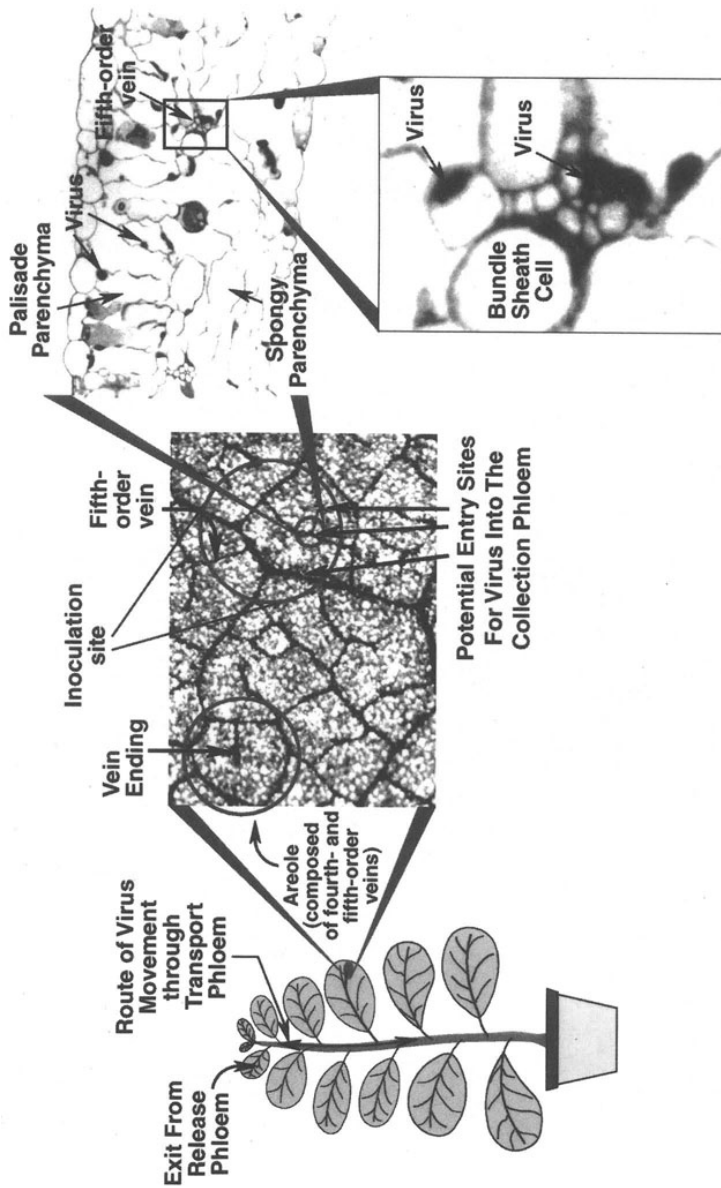


Fig. 1. Minor vein architecture and cell structure of exporting leaves representative of the three main types observed in dicotyledonous plants. Micrographs are oriented such that adaxial (top) portion of leaf would be at top of each panel. Left. Abaxial minor vein from *Cucumis melo*. Note the electron-dense cytoplasm and fragmented vacuoles in intermediary cells (I). Also, note that the abaxial bundle sheath (BS) cell abuts a sieve element. Center. Abaxial minor vein from *Nicotiana benthamiana*. Note alternating ring of vascular parenchyma (VP) and companion (CC) cells around single sieve element. Companion cells, like intermediary cells in *C. melo*, are rich in cytoplasm and contain fragmented, small vacuoles. Bar = 2  $\mu$ m. Right. Minor vein from *Pisum sativum*. Note lack of symmetry in position of cell types for this vein compared with vein from *N. benthamiana*: some plant species have very high proportions of nontypical vein architecture. Note cell wall invaginations (white protrusions) in transfer (T) cells that are a hallmark of this cell type. Bar = 2  $\mu$ m. X, Xylem tracheary element. (Micrographs supplied by X.S. Ding)



**Fig. 2.** Plant inoculated with tobacco mosaic virus (TMV) and *enlargements* showing veins in an inoculated, mature leaf of *Nicotiana tabacum*. Arrows pointing to black spots in the two *right-hand panels* (transverse sections) indicate examples of locations of virus in these cells as determined by immunocytochemistry with an antibody against the coat protein followed by silver enhancement. (Micrographs showing leaf minor veins and immunocytochemical localization of TMV CP supplied respectively, by P. Derrick and X. S. Ding)

spongy parenchyma cells (e.g. *Populus*, Russin and Evert 1985; *Coleus*, Fisher 1986; *Solanum*, McCauley and Evert 1989). In *Populus* (Rusin and Evert 1985), *Coleus* (Fisher 1986), *Cananga* (Fisher 1990) and *Moricandia* (Beebe and Evert 1992) the plasmodesmal densities between the MCs are markedly higher than in *Solanum* (McCauley and Evert 1989), *Spinacia* (Warmbrodt and Van der Woude 1990) and *Vicia* (Bourquin et al. 1990). In *Populus*, *Coleus* and *Moricandia*, a distinct layer of poorly defined MCs appears in the plane of the minor veins (Rusin and Evert 1985; Fisher 1986; Beebe and Evert 1992). The plasmodesmal density between these cells and the adjacent MCs and BSCs is significantly higher than those in the palisade and spongy MC domains. Although these cells lack the structural specialization of the paraveinal MCs, which unite all of the vascular bundles horizontally in many legumes (e.g. Franceschi and Giaquinta 1983a), the plasmodesmal configuration and density between these cells and their strategic location suggests an intermediary role for them in photosynthate collection and processing. At the end of the mesophyll track, the plasmodesmal densities between MCs and BSCs are not significantly different from those between the MCs. Certainly, it would be interesting to determine if viruses move most quickly between the highly connected cells in such symplasmic domains.

The intercellular pre-SE pathway is further distinguished between the species in that, particularly at the interface between the BSC/VPC domain and the SE/CC domain, the number of PD differs greatly. In the highest-order veins, the number of PD connecting these two domains can differ by a factor of 1000 between the dicotyledonous species (Gamalei 1989; also see Van Bel and Gamalei 1992; Gamalei et al. 1994; Grusak et al. 1996 for reviews). In species where few PD occur between the SE/CC complex and other cells, the number of PD between the MCs is also generally low (Gamalei 1989, 1990).

The degree of symplasmic connectivity between the SE/CC complex and other cells coincides with the structure of the CCs (Fig. 1). In plants where the symplasmic connectivity is high at this interface, the CCs in the minor veins, now called intermediary cells (ICs), contain vesicular labyrinths that may be endoplasmic reticulum (Gamalei et al. 1994). These vesicular labyrinths appear to fluctuate in volume with the transit of photosynthate (Gamalei et al. 1994). When the symplasmic connectivity between CCs and other cells (besides the SEs) is very low, the CCs in the minor veins, now called transfer cells (TCs), possess cell wall invaginations varying in volume with the transit of photosynthate (Wimmers and Turgeon 1991; Gamalei et al. 1992). Intermediate to this, but more closely allied with TCs in regard to symplasmic conductivity, are the smooth-walled CCs (Gamalei 1989; Turgeon et al. 1993; Turgeon 1996).

Major difficulties in depicting a consistent image of the phloem loading zone are the qualitative and quantitative differences between cells in vein endings and the veins encompassing an areole (see Fig. 2 for locations of these veins). Intuitively, one expects that phloem loading takes place in the vein endings and the highest-order veins. Surprisingly, the vein endings protruding into the areoles often lack SEs (Fisher and Evert 1982; McCauley and Evert 1989; Fisher 1990) or have sieve tube endings at various intermediate points before the vein ends (Lersten 1990; Horner et al. 1994). Species with few or no sieve tubes in the terminal veins have smaller areoles which may have implications for photosynthate entry into the vascular system (Horner et al. 1994) through the maintenance of a specific pre-SE trajectory length.

As for the vein orders that encompass the areole, differences in phloem anatomy have been observed (McCauley and Evert 1989; Fisher 1990). In *Solanum tuberosum* and *Nicotiana tabacum*, for instance, only abaxial SEs occur in the seventh- and fifth-order veins respectively (Ding et al. 1988; McCauley and Evert 1989; X. S. Ding et al. 1995, Fig. 2), whereas adaxial phloem is also present in the sixth- and second-order or lower-order veins (Ding et al. 1988; McCauley and Evert 1989). To further complicate an assessment of the loading pathway, several dicotyledonous species (e.g. from Acanthaceae, Cucurbitaceae, Lamiaceae, Solanaceae, Rosaceae) possess different types of SE/CC complexes in the minor veins and these complexes are symplasmically connected with the mesophyll domain in a disparate fashion (Turgeon et al. 1975; Fisher 1986; Schmitz et al. 1987; McCauley and Evert 1989; van Bel et al. 1992).

### β) *Monocotyledons*

In leaves of monocotyledons, the vein system radiates from the leaf base and the major veins are mostly parallel to one another through the leaf blade. The vein system consists of transverse veins – the smallest units of the veinal network – and small, intermediate and large longitudinal vascular bundles. The function of transverse veins in phloem loading is a matter of debate, as some research indicates they are buffering storage compartments enabling coordinated translocation in the longitudinal veins and thus not primary phloem loading zones (Lush 1976).

As in dicotyledons, the pre-SE trajectory is highly variable. Within the Gramineae (grasses), which is the only family for which the leaf anatomy of many species has been studied in detail, this variability is most evident (e.g. Lush 1976; Cartwright et al. 1977; Evert et al. 1977; Colbert and Evert 1982; Chonan et al. 1985; Russell and Evert 1985; Botha and Evert 1988; Eastman et al. 1988a,b; Dannenhoffer et al. 1990; Robinson-Beers and Evert 1991a,b; Botha 1992; Botha and van Bel 1992). The little data available in the literature indicate relatively high PD frequencies in the



MC region (Robinson-Beers and Evert 1991b). The sheath region around the vascular bundles is especially variable between vein orders and between plant species. A vein sheath is absent or is composed of one, two or three layers which may be of procambial (mestome sheath) or parenchymatous (bundle sheath) origin (Dengler et al. 1985; Eastman et al. 1988a; Bosabalidis et al. 1994). The mestome sheath cell walls are always lignified, while the BSCs are often lignified (Eastman et al. 1988a). Both sheath cell types may have suberized depositions in their cell walls, and this is most likely to be observed in the tangential walls (e.g. Kuo et al. 1974; Evert et al. 1977; Eleftheriou and Tsekos 1979; Eastman et al. 1988a).

Another striking feature of the grasses is the high plasmodesmal frequency between the non-vascular cells and the low, in many instances non-existent, symplasmic connectivity between the vascular cells (*Themeda*, Botha and Evert 1988; *Eragrostis*, *Panicum*, *Bromus*, Botha 1992; Botha and van Bel 1992). Low plasmodesmal frequencies between the vascular cells also occur in the Commelinaceae (van Bel et al. 1988).

Within the metaphloem of smaller vascular bundles, two types of SEs occur in Gramineae (Kuo and O'Brien 1974; Miyaka and Maeda 1976; Cartwright et al. 1977; Walsh 1974; Evert et al. 1978; Colbert and Evert 1982; Botha 1992) and Commelinaceae (van Bel et al. 1988). The thin-walled SEs differentiate before the thick-walled SEs which lie closest to the xylem vessels (Walsh 1974; Evert et al. 1978; van Bel et al. 1988; Botha 1992). In *Commelina* (van Bel et al. 1988) and sugar cane (Robinson-Beers and Evert 1991a), the thin-walled SEs in the metaphloem are mainly symplasmically linked with a CC, whereas the thick-walled SEs are mainly associated with a VPC. In the transverse veins, the single SE is most often categorized as "thin-walled" (Colbert and Evert 1982; Russell and Evert 1985; Dannenhoffer et al. 1990), although the P-plastids are most like those in thick-walled SEs (Colbert and Evert 1982). The proportion between thick- and thin-walled SEs shifts with the vein order. In the small longitudinal bundles the ratio of thin-walled to thick-walled SEs is much lower (between 1 and 3) than that observed in the large longitudinal bundles (between 3 and 10; Colbert and Evert 1982; Russell and Evert 1984; Dannenhoffer et al. 1990).

The small and/or intermediate bundles are considered to be the major sites of phloem loading in grasses for several reasons. Firstly, the mestome sheath of the intermediate bundles in wheat leaves has more pits and considerably larger pit areas than those encountered in the other bundles (Kuo et al. 1974). Secondly, <sup>14</sup>C-labelled photosynthate was collected mainly in the minor veins of *Panicum* (Lush 1976). Likewise, <sup>14</sup>C-photosynthate was accumulated in the thin-walled SEs of the small and intermediate bundles in maize (Fritz et al. 1989). The transverse veins do not seem to be involved actively in phloem loading. The transverse veins were symplasmically isolated from the MCs in wheat (Kuo et al. 1974)

and were heavily labelled with  $^{14}\text{C}$  only sometime after the main pulse of  $^{14}\text{C}$  had passed out of the leaves of *Panicum* (Lush 1976). Transverse veins may therefore be engaged in storage and may be able to deviate photosynthate streams to coordinate the functioning of the different longitudinal veins (Lush 1976). Lateral transfer of assimilates from smaller to larger veins via the transverse veins was evidenced by microautoradiography in wheat (Altus and Canny 1982) and maize (Fritz et al. 1989). Shunting the photosynthate through the transverse veins finally resulted in the collection of photosynthate in the large bundles which function exclusively for export (Kuo et al. 1974; Lush 1976; Altus and Canny 1982; Fritz et al. 1989).

### c) Ontogeny of the Minor Vein System in Relation to Transition of Leaf from Sink to Source Tissue

#### $\alpha$ ) *Dicotyledons*

The ontogeny of the minor vein system and the concurrent sink-source transition in dicotyledonous leaves have been comprehensively reviewed by Turgeon (1989). The network of veins develops initially from the leaf base. During this stage, only the lower-order (i.e. larger) veins differentiate, giving the leaf its characteristic vein pattern. The initial differentiation goes up to the third-order veins in tobacco (Turgeon 1987) or the fourth-order in sugar beet (Schmalstig and Geiger 1987). After completion of the major vein system, the structural and functional maturation of the higher order (i.e. smaller or minor) veins begins and proceeds in the basipetal direction (Turgeon 1989). The minor vein pattern is variable between individual leaves and the final pattern exhibited may depend on environmental factors experienced by the developing cells.

The second wave of differentiation involving the minor veins proceeds in a narrow, distinctly demarcated band (Schmalstig and Geiger 1987; Turgeon 1987). In this band, PD between the importing larger veins and the surrounding tissue are likely truncated (Ding et al. 1988; Bourquin et al. 1990). Simultaneously, the highest-order veins, responsible for photoassimilate export, differentiate (Roberts et al. 1997). In the newly developed veins, sucrose carriers develop; this was visualized by expressing the marker protein, GUS, behind the promoter (AtSuc2) of a sucrose carrier in *Arabidopsis thaliana* (Truernit and Sauer 1995). A similar finding was obtained for the StSUT1 and PmsUC2 sucrose transporter, from potato and *Plantago*, respectively, by RNA gel blots or RNase protection analyses of source and sink tissue (Riesmeier et al. 1993; Stadler et al. 1995). It is within this narrow band that the leaf irreversibly switches from an importer to an exporter of photosynthate (reviewed in Grusak et al. 1996). The irreversible nature of the change

was demonstrated by the findings that source tissue of darkened tobacco leaves or albino leaves of tobacco mutants grafted to a green tobacco stock could not import significant quantities of photoassimilates (Turgeon 1984, 1986). Also, the minor veins imported no photosynthate, indicating they do not have this capability.

The sink/source or import/export transition is correlated with the development of the minor vein network. In the *Cucurbita* for instance, import into the lamina tip stops when the blade is 10% expanded. The base of the lamina stops importing as soon as the blade is 45% expanded. The small amount of material initially exported from the leaf tip is redistributed to the still importing leaf base, delaying export from the lamina until the blade is 35% expanded (Turgeon and Webb 1973).

Photosynthate unloading in the sink zones of the leaf likely is symplasmic. Photosynthate unloading was insensitive to p-chloromercuribenzenesulphonic acid (PCMBS; Schmalstig and Geiger 1985) and anoxia (Turgeon 1987). Insensitivity of treatments that interfere with transmembrane transport of sugars indicates that transport via the apoplast does not contribute significantly to phloem unloading. A morphometric analysis of the importing veins supports a symplasmic mode of phloem unloading. During the sink to source transition of the leaf tissue, the number of PD along the presumptive unloading pathway decreased considerably (Ding et al. 1988). Symplasmic phloem unloading should allow viruses to freely invade the growing zones of the leaves, until the sink to source transition takes place. This view has been substantiated by a recent study in which 5,6 carboxyfluorescein diacetate (CFDA) distribution and the pattern of GFP-labelled potato X virus were compared in *Nicotiana benthamiana* leaves proceeding through a sink source transition (Roberts et al. 1997; for further discussion see Sect. 6).

### β) Monocotyledons

The meristematic progeny of the vein system, the architecture of the vein network and the vein anatomy are very different between monocotyledons and dicotyledons. If, however, the few data available are representative for the monocotyledons (see Dannenhoffer and Evert 1994 and references therein), the physiological events associated with the sink/source transition look surprisingly similar in both groups. The large vascular bundles differentiate as procambial strands lacking continuity with the stem vasculature during the early development of the leaf. As in dicotyledons, the vein differentiation in the primordium takes place in two waves: an acropetal differentiation of the major veins and subsequent basipetal differentiation of the smaller veins (Dannenhoffer and Evert 1994). The leaf primordium acts as an importer of assimilates. As the development proceeds and supplementary veins differentiate in

the tip of the leaf, the apex begins to export photosynthates, which are used partly for its own growth and partly for export to other plant parts (Anderson and Dale 1983). Again, as in dicotyledons, the onset of photosynthate export concurs with the emergence of the veins responsible for phloem loading (Kuo et al. 1974). A major distinction in the sink physiology between monocotyledonous and dicotyledonous leaves may lie in the fact the monocotyledonous sink leaves appear to unload apoplasmically. The SEs in the protophloem which function during the import stage are absolutely symplasmically isolated from the adjoining cells (Evert and Russin 1993). Thus, in contrast to the situation in dicotyledons, viruses moving through the PDs should not have symplasmic access to MCs during the importing stage in monocotyledons. During the exporting stage virus infection may be possible via the thick-walled SEs and the associate VPCs which are symplasmically coupled to the BSCs and the MCs (van Bel et al. 1988; Robinson-Beers and Evert 1991a).

#### d) Physiology of the Phloem Loading Zone

##### $\alpha$ ) *Dicotyledons*

As pointed out in Section 2.a. $\alpha$ , the plasmodesmal densities in the MCs of certain genuses (*Populus*, *Coleus*, *Cananga*, *Moricandia*) are clearly greater than those in others (*Solanum*, *Spinacia*, *Vicia*). The first group includes representatives of families with a symplasmic minor vein configuration, except for *Moricandia* (Brassicaceae), while the second group belongs to families with an apoplasmic minor vein configuration (Gamalei 1989). This suggests that plants with low symplasmic connectivity between the MCs are more likely to be apoplasmic species, as reported by Gamalei (1990). The plasmodesmal densities at the respective interfaces suggest that the photosynthate is not directed radially (i.e. directly) to the minor veins. The plasmodesmal configuration in several species (*Populus*, *Coleus*, *Moricandia*) indicates that photosynthate may be collected by the MCs in the plane of the minor veins and then is transported horizontally to the BSCs in the phloem region (Rusin and Evert 1985; Fisher 1986; Beebe and Evert 1992). A similar function, though more specialized, was attributed to the paraveinal MCs in soybean (Franceschi and Giaquinta 1983b).

Those BSCs to which the photosynthate may be predominantly directed are symplasmically linked with the CCs in the minor veins. Experiments with stripped leaf discs and intact leaves of more than 40 species demonstrated that the ultrastructural configuration of the minor veins reflects the mode of phloem loading (Turgeon and Wimmers 1988; van Bel et al. 1992; Flora and Madore 1996). In "apoplasmic" species, PCMBs drastically impeded phloem loading, whereas phloem loading

was essentially unaffected by PCMBs in "symplasmic" species (van Bel et al. 1992, 1994). On the basis of these observations, the symplasmic configuration seems to offer a much wider corridor for virus movement. No clarity exists regarding the mode of phloem loading in species with a mixed vein configuration (Gamalei 1990; van Bel et al. 1992; van Bel 1993). The pathway of phloem loading may switch with the environmental conditions (van Bel 1993). Certainly, the preferential passageway for viruses would be the symplasmic one, if both pathways are operating in parallel.

### β) *Monocotyledons*

In view of the lignification and suberization of the sheath walls in grasses, the conduit for photosynthate transport from MCs to the phloem is expected to be symplasmic. This is consistent with the high plasmodesmal frequencies in the tangential walls of the parenchymatous bundle sheath and mestome sheath (Botha and Evert 1988; Robinson-Beers and Evert 1991a; Botha 1992). On the other hand, the apoplastic marker trisodium 3-hydroxy-5,8,10-pyrenetrisulphonate, applied via the xylem, moved outward into the surrounding tissues of the veins of all orders in ten gramineous species (Eastman et al. 1988b). The suberized frame may allow the water molecules to move apoplastically toward the water-destitute leaf cells and simultaneously force the photosynthate to move symplasmically to the SE/CC complexes (Evert et al. 1985). Most likely, photosynthate is symplasmically channeled to the mestome sheath cells that abut the metaphloem in wheat (Kuo et al. 1974). In these regions, plasmodesmal frequencies on the periclinal walls are abundant. In barley, so-called S-type BSCs were identified in the region adjoining the metaphloem and part of the xylem (Williams et al. 1989). These cells flanking the metaphloem at both sides may play a central role in the regulation of the photosynthate transit from MCs to phloem.

The scanty symplasmic connectivity between the BSCs and the phloem elements, excluding the VPCs (Evert et al. 1978; Botha and Evert 1988; Botha 1992; Botha and van Bel 1992), and within the phloem region itself (Botha and Evert 1988; Botha 1992; Botha and van Bel 1992) suggests apoplastic loading in the Gramineae (Evert et al. 1977). Phloem loading is probably executed by the thin-walled SEs exclusively as was demonstrated by microautoradiography (Cartwright et al. 1977; Fritz et al. 1983). Additional evidence for this comes from plasmolytic studies where it was determined that osmotic potentials were much lower, and thus sugar concentrations much higher, in thin-walled SEs than in thick-walled SEs (Evert et al. 1978). In *Commelina*, the symplasmic connection between the thick-walled SEs and the mesophyll was hypothesized to provide a symplasmic pathway of phloem loading paral-

lel to the apoplasmic one into the thin-walled SEs (van Bel et al. 1988). Thick-walled SEs, however, may not be involved in phloem loading (Cartwright et al. 1977; Evert et al. 1978; Fritz et al. 1983). Instead, the thick-walled SEs may play a role in the temporary storage of photosynthate and in the transfer of materials from xylem vessels to the phloem and vice versa in maize leaves (Fritz et al. 1983).

In regard to virus movement, the ultrastructural differences of the PD throughout the presumptive pathway from MCs to SEs need to be recognized (Robinson-Beers and Evert 1991b). The question emerges as to whether the PD at each cellular interface present a different barrier to a particular virus, each interface requiring different responses by the virus to allow its movement.

## e) Functional Domains and Physiology of Transport Phloem

### $\alpha$ ) *Dicotyledons*

In dicotyledons, the vascular bundles are essentially circularly arranged with a cambium between phloem and xylem. Radially oriented sheets of VPCs (rays) enable solute exchange between phloem and xylem (reviewed by van Bel 1990). The few studies on the plasmodesmal frequencies between SEs and the adjoining tissue in transport phloem indicate a symplasmic constriction at the interface between SE/CC complex and phloem parenchyma cells (PPC). Limited numbers of PD occur at the interface between the SE/CC complex and PPCs. In the primary phloem of *Phaseolus* stems, the protophloem SEs are fully isolated from the neighbouring cells, whereas the metaphloem SEs display limited symplasmic connectivity with the PPCs (Wood et al. 1997). The same applies to SEs in the secondary stem phloem of *Phaseolus* and *Ricinus* internodes (Hayes et al. 1985; van Bel and Kempers 1991) and *Cucurbita*, *Lythrum*, *Vicia* and *Zinnia* stems (Ammerlaan et al. 1996).

The branched PD between the SE and CC or pore/plasmodesma units (PPU, van Bel 1996a) seem to allow lucifer yellow (LYCH) movement under all conditions (van der Schoot and van Bel 1989; van Bel and Kempers 1991; Oparka et al. 1992; van Bel and van Rijen 1994). The PPUs have exclusion diameters larger than 10 kDa, most likely in the 20–30-kDa range (Kempers et al. 1993; Kempers and van Bel 1997), which would permit movement of most intact P-proteins (reviewed by van Bel and Kempers 1997). The significance of the large passageways for virus movement through the PPUs is, as yet, unclear. Despite the broadness, viruses may need movement proteins to pass the PPU-corridors (see discussion of this in Sect. 5b).

Fluorochromes intracellularly injected into the SEs in tangentially cut stem or stolon slices (van der Schoot and van Bel 1989; van Bel and

Kempers 1991; Oparka et al. 1992; van Bel and van Rijen 1994) and in intact tomato petioles (Rhodes et al. 1996) moved exclusively through the SE/CC complexes. Symplasmic isolation of the SE/CC complex was supported by the dispersal pattern of CFDA, applied to the cotyledons of *Arabidopsis* seedlings. The dye remained confined to the phloem, until being released in the growing zone of the root tips (Oparka et al. 1994, 1995a). In the latter area, the size exclusion limit (SEL) of the PD in the symplasmic path between SE and sink cells (barley, Warmbrodt 1985b; maize, Warmbrodt 1985a; cucumber, Warmbrodt 1986) can be modulated by osmotic stress (Schulz 1995). Apparently, the few PD between SE/CC complexes and PPCs in transport phloem are usually closed, except for those in the release phloem of the roots, where a strong symplasmic continuity between SEs and surrounding tissues seems to exist (Oparka et al. 1994, 1995a).

Contrasting the evidence for symplasmic discontinuity between CCs and PPCs, other experiments indicate a reversible and controlled gating of the PD between SE/CC complexes and PPCs in transport phloem (Hayes et al. 1987). In stems of summer-grown bean plants with a high source-sink ratio, photosynthate appeared to move symplasmically from the sieve tubes into the surrounding tissues, while photosynthate was apoplastically released from the phloem in winter-grown plants with a low source-sink ratio (Hayes et al. 1987). The different pathways of photosynthate release have recently been substantiated by experiments with CFDA (Patrick and Offler 1996). Further experiments with *Arabidopsis* seedlings showed that the metabolic status of the stem tissue affected the gating of these PD. CFDA moved out from transport phloem being treated with metabolic inhibitors (Wright and Oparka 1997). This phenomenon is consistent with the observation that metabolic poisons open up PD and thus allow a higher degree of symplasmic exchange (Tucker 1993; Cleland et al. 1994). Possibly, lateral spread of viruses from the transport phloem may be modulated by the physiological condition of the host (see a further discussion of this topic in relation to virus movement in Sect. 5.b).

### β) *Monocotyledons*

In monocotyledons, the vascular bundles are dispersed throughout a ground tissue and do not display secondary growth. To our knowledge, no data are available on the plasmodesmal frequencies between the SE/CC complexes and the neighbouring phloem elements in petioles and stems. As for the major veins, a narrow symplasmic trajectory leads from the thick-walled SEs via the VPCs to the BSCs in rice (Chonan et al. 1985) and from the thin-walled SEs via the CCs and VPCs to the BSCs in maize (Robinson-Beers and Evert 1991b).

The scarce physiological data indicate a symplasmic continuity between the SE/CC complexes and the parenchyma cells surrounding the vascular bundles in sugar cane stalks (Jacobsen et al. 1992; Welbaum et al. 1992). The vascular bundles are enclosed by a sclerenchymatic bundle sheath and the phloem is surrounded by a lignified barrier in the apoplast (Jacobsen et al. 1992). In older stalks, the walls of the parenchyma cells surrounding the vascular bundles become suberized (Jacobsen et al. 1992). Perfusion experiments with the apoplastic tracers trisodium 3-hydroxy-5,8,10-pyrenetrisulphonate (Jacobsen et al. 1992) and trisodium 8-hydroxy-1,3,6-pyrenetrisulphonate (Welbaum et al. 1992) confirmed that this water-impermeable collar did not allow water movement out of the xylem. These observations were strongly indicative of radial symplasmic photoassimilate transport, increasing in comparison with radial apoplastic movement with the age of the stalk. As a matter of fact, sugars were still being accumulated in the storage parenchyma, while lignification and suberization proceeded (Jacobsen et al. 1992).

### 3. Transport of Virus to Vascular Tissue

#### a) Into and Between Mesophyll Cells

Virus transport between MCs is a heavily studied area with multiple recent reviews (Maule 1991; Deom et al. 1992; Citovsky and Zambryski 1993; Carrington et al. 1996; Gilbertson and Lucas 1996; Sanderfoot and Lazarowitz 1996). As such, this chapter will only discuss aspects of virus cell-to-cell movement that relate to vascular-dependent accumulation.

Mechanical inoculation of leaves with viruses that are not limited to vascular tissue leads to infection in epidermal and possibly palisade (adaxial surface of leaf) or spongy (abaxial surface of leaf) parenchyma cells (Fry and Matthews 1963; Wilson et al. 1990; Fig. 2). For these viruses, disassembly of the structure encasing the viral genetic material (i.e. the capsid) must take place in these cells.

It recently was shown that more than 70% of the tobacco mosaic tobamovirus (TMV) genome is freed from the capsid from the 5' end toward the 3' end of the RNA within 3 min of entry into a tobacco cell protoplast (Wu et al. 1994). By 30 min, the viral genome is totally released after further uncoating in the 3'-5' direction (Wu and Shaw 1996). By 40 min post-infection, progeny virus particles begin to appear (Wu et al. 1994). Results from older studies generally support these findings in that partially uncoated viral translation complexes were identified within 60 min of inoculation of leaves from *Nicotiana tabacum* with TMV or potato potexvirus X (PVX) (Shaw et al. 1986; Wilson and Shaw 1987). Therefore, virus replication can be an extremely rapid process. If a



virus can produce progeny encapsidated virus in ~ 1 h per cell, and most cells are no more than six cells from vascular tissue, TMV should reach vascular tissue in ~ 6 h (see Fig. 2 for position of minor veins in mature leaf of *N. tabacum*; also see Mauseth 1988; Leisner and Turgeon 1993). In fact, these results could be interpreted as conservative estimates of the time when TMV is competent for cell-to-cell movement since the viral protein(s) necessary for this movement accumulates maximally before the coat protein (CP) for encapsidation of the viral genome in protoplasts (compare results from Derrick et al. 1997; Siegel et al. 1978; Watanabe et al. 1984). It is therefore surprising that systemic infection is not observed after infection with TMV until 32–48 h post-inoculation at 24 °C (Oxelfelt 1970) or with cucumber mosaic cucumovirus (CMV) until 24–30 h post-inoculation (Gal-On et al. 1994). For the DNA virus, cauliflower mosaic caulimovirus (CaMV), a period > 3 days post-inoculation (dpi) is necessary for a sufficient number of virus particles to exit the inoculated leaf to induce systemic symptoms (Melcher 1989; Leisner et al. 1992). All these experiments were conducted by detaching inoculated leaves at various times post-inoculation and observing the remainder of the plant for systemic symptoms.

Interpreting the impact of the results from protoplast infections and leaf detachment assays on vascular-dependent accumulation is difficult. Firstly, the rate of accumulation of progeny virus in protoplasts may not reflect the rate of accumulation of virus in intact tissue. Secondly, the rate of viral cell-to-cell movement cannot be estimated from protoplast studies. Lastly, viruses may have moved prior the leaf detachment, but in quantities below a threshold necessary to establish a systemic infection.

Thus, experiments utilizing different procedures to study vascular-dependent spread of viruses are necessary to support conclusions derived from the protoplast and leaf detachment experiments. Cell-to-cell movement of viruses in intact tissue has been estimated in several studies. Fry and Matthews (1963) did not detect movement of TMV from epidermal cells to MCs until 4 h post-inoculation. Through microinjection studies followed by trichome detachment, the time of movement of tobacco rattle tobnavirus (TRV) from an initially injected leaf trichome cell to another trichome cell was shown to be at least 4 h post-inoculation (Derrick et al. 1992). These researchers also showed that SELs of PD in these cells, a possible indicator of cell receptivity for virus movement, were not modified for at least 2 h post-inoculation. Thus, from these studies a delay in movement out of epidermal cells beyond what might be expected from the protoplast work is apparent. Interestingly, subsequent movement of TRV into the second and third cells occurred at approximately 5 and 7 h post-inoculation, respectively (Derrick et al. 1992). These researchers speculated that the damage incurred by microinjection and the subsequent time for recovery of the injected cell were not the cause of the delay of movement to the next cell; they noted that

the presence of the viral factors necessary for moving virus cell-to-cell or for establishing infection in the second cell, which would not be present initially in the first cell, may be responsible for the observed increased rate of cell-to-cell movement. One such factor may be the 30-kDa movement protein (MP) of TMV which increases the SEL of PD several cells from the injection site (Waigmann et al. 1994). Fujiwara et al. (1993) determined that the related 35-kDa MP of red clover necrotic mosaic dianthovirus (RCNMV) appears to move cell-to-cell after microinjection. Whether the TMV MP itself moves or induces a signal sensed by neighbouring cells is unknown, but regardless of the mechanism, its presence could certainly potentiate cells for rapid virus spread. Apparently then, the delay in systemic virus accumulation is at least partially due to delayed infection of MCs from the first infected cell.

Other potential causes of delayed systemic virus accumulation include delays in the infection of vascular cells and transport of virus into SEs, but probably not movement through SEs. As will be discussed in detail in Section 5a, most phloem-transmitted viruses move rapidly through the sieve tubes at rates in the centimetres per hour range (reviewed in Bennett 1956); thus, this portion of the system does not limit the rate of virus accumulation in systemic tissue under normal phloem flux conditions. Further support for this postulate comes from the previously mentioned experiments where systemic spread of virus was measured by detaching inoculated leaves at various times post-inoculation (Oxelfelt 1970; Gal-On et al. 1994). In these studies, virus would only have had to move through a short section of the transport tube (i.e. from the entry point into an SE to the sieve tube in the petiole of the inoculated leaf) to establish a systemic infection. The fact that they could not do so at or before 24 h suggests that factors limiting spread exist in the inoculated leaf. It therefore appears that, in addition to a delay in infecting a second cell after inoculation, some delay in systemic virus accumulation is associated with the vascular tissue of the inoculated leaves. This delay, as well as any delay manifested in systemic tissue, may be due to a block in movement through particular vascular cells or to a restriction in accumulation in particular vascular cells leading to a lower titer of virus available for spread and a subsequent delay in detectable systemic virus accumulation.

#### b) Mesophyll Cell to Bundle Sheath Cell

Little research has been directed at studying virus movement between MCs and BSCs, but the research that has been conducted indicates this is not a special boundary for virus transport. Through microinjection studies with purified TMV MP produced in *E. coli*, it was shown that the SELs of PD between the MCs and BSCs of third-order veins (i.e. major

veins) from mature leaves of *N. tabacum* were altered similarly to those between MCs (Ding et al. 1992). In studies with two strains of TMV, both viruses were present in similar percentages of MCs and BSCs of fifth-order veins (i.e. minor veins) from mature leaves of *N. tabacum* at 3 dpi, a time when systemic infection is occurring for this virus (X. S. Ding et al. 1995). Thus, the results for TMV indicate that the PD between MCs and BSCs do not pose a significant barrier for movement of this virus. Similar analyses should be conducted with other viruses to determine whether this conclusion is widely applicable. Mutants of viruses should also be studied to determine if viral proteins other than the MPs of these viruses regulate movement. Lastly, host plants resistant to systemic infection should be analysed to determine if the resistance is associated with a block in accumulation in BSCs.

As noted in Section 2.a, we have made the assumption that viruses invade minor veins for vascular transport to systemic tissue. This has yet to be firmly proven, but some evidence exists to support this assumption. Leisner et al. (1992) observed a pattern of lesions on the inoculated leaf lamina suggesting that CaMV invades the vascular system through minor veins. Also, minor veins make up more than 90% of the vasculature in mature leaves and would be the first veins virus would contact during cell-to-cell movement. (X. S. Ding et al. 1995; Grusak et al. 1996; see Fig. 2 for an example of the anatomy and position of minor veins in *N. tabacum*). These veins are irreversibly modified in maturing leaves to function solely in photoassimilate export (see Sect. 2.c for a further discussion of this). In spite of this supportive information, definitive studies must be undertaken to prove that viruses utilize these veins for entry and transport to other parts of the plant.

#### 4. Transport of Virus Into and Within Vascular Tissue of Inoculated Leaves

##### a) Bundle Sheath Cell to Vascular Parenchyma and Companion Cells

Transport of virus from BSCs to VPCs and/or CCs within minor veins is limited for some viruses. In *Nicotiana tabacum*, the percentage of internal vein cells (i.e. VPCs and CCs) infected by both an attenuated masked (M) and a severe (U1) strain of TMV at 3 dpi was less than the percentage of BSCs infected (X. S. Ding et al. 1995). The CCs showed the most prominent difference in infection, with only ~10–30% of the cells infected compared with nearly 90% of the BSCs. Interestingly, for the U1 strain, the percentage of VPCs infected was similar to the percentage of BSCs infected. Thus, the PD between the BSCs and VPCs apparently do not limit transport of this virus. However, the percentage of VPCs and CCs infected by the M strain were significantly decreased compared with

the U1 strain. These results indicate that the M strain of TMV has difficulty traversing the boundary between the BSCs and the VPCs or CCs. This may partially account for the delayed phloem-dependent accumulation phenotype displayed by the M strain compared with the U1 strain. An inability to replicate in VPCs or CCs does not appear to be the cause of this decrease since all the VPCs and CCs would be at a similar developmental stage in these leaves and some VPCs and CCs accumulated substantial amounts of virus and viral replicase components (X. S. Ding et al. 1995).

Support for a control point in virus movement from BSCs into VPCs and CCs comes from various studies. In microinjection studies, the MP of TMV did not alter the SELs of PD between BSCs and VPCs of third-order veins from leaves of *N. tabacum*, although it did increase the SELs of PD between MCs and BSCs (Ding et al. 1992). This result clearly demonstrates that the PD between the BSCs and VPCs are not identical to those between MCs and MCs and BSCs. Other studies with phloem-limited viruses have also determined that the boundary between the BSCs and internal vascular cells is different as evidenced by the limited ability of these viruses to accumulate in BSCs or MCs surrounding the infected VPCs and CCs (D'Arcy and de Zoeten 1979; Shepardson et al. 1980; Sanger et al. 1994; van den Heuvel et al. 1995; see Sect. 6). Very recently it has been determined that the expression of the gene encoding the 2a protein of CMV in transgenic tobacco plants protects these plants from challenge virus by preventing virus entry into VPCs and CCs from BSCs of minor veins (Wintermantel et al. 1997).

Recently, host factors that regulate fluxes of photosynthate or viruses between the BSCs and VPCs or CCs have been genetically and physiologically characterized. A maize mutant was characterized wherein only the PD between the BSCs and VPCs in minor veins were structurally modified (Russin et al. 1996). The aberrant structures were correlated with a lack of phloem-loading capacity, thus identifying a critical role for VPCs in photosynthate transport. A single locus controls the host phenotype. This boundary between BSCs and internal vascular cells has also been determined as critical for infection of soya-bean by cowpea chlorotic mottle bromovirus (CCMV). CCMV did not accumulate in VPCs or CCs within the bundle sheath of a resistant soya-bean line (PI 346304; Goodrick et al. 1991).

The results from studies reviewed in the preceding two paragraphs indicate that unique sets of viral and host factors control virus entry into VPCs and/or CCs. The viral factor for TMV is most likely the 126-kDa and/or 183-kDa proteins of TMV (X. S. Ding et al. 1995; Derrick et al. 1997). It is unlikely to be the coat protein (CP) since TMV lacking CP can enter VPCs (Ding et al. 1996). Interestingly, for CCMV the viral factor for differential systemic accumulation in PI 186465 of cowpea maps to RNA1 of the virus (Wyatt and Kuhn 1980). RNA1 contains the analo-

gous open reading frame to the TMV open reading frame (orf) encoding the 126-kDa protein. These proteins are known to modulate viral replication activity and contain sequences with similarities to those in methyltransferases and helicases. Whether these activities or other uncharacterized activities affect movement between BSCs and the VPCs or CCs requires further study. The host protein(s) in soya-bean line, PI 346304, controlling accumulation of CCMV in VPCs and CCs is unidentified. Host factors specifically controlling vascular-dependent accumulation in other virus/host systems have been genetically characterized, but their identities are unknown and the cellular site of their effect rarely has been studied (Holmes 1955; Kuhn et al. 1981; Lei and Agrios 1986; Barker 1987b; Dufour et al. 1989; Law et al. 1989; Simon et al. 1992; Leisner et al. 1993; Murphy and Kyle 1995; Schaad and Carrington 1996; see Sect. 4.b).

In some cases the host resistance decreases accumulation of virus in inoculated leaves, making it difficult to determine whether vascular-dependent virus accumulation is decreased directly due to the lack of a host factor necessary for vascular-dependent movement or indirectly through a virus-induced host resistance followed by decreased cell-to-cell movement. This difficulty in determining whether host resistance factors are simply unable to support movement or actively repress it was recently demonstrated for *Arabidopsis* ecotypes resistant to systemic infection by CaMV (Callaway et al. 1996). It was determined that the pattern of accumulation of normal virus in an inoculated leaf of a resistant host was similar to that of a movement defective virus. Although one could speculate that the resistance gene prevented movement simply by no longer supporting virus movement, these researchers also found that pathogenesis-related proteins and a phytoalexin were induced, thus indicating an active host response which may repress movement. This information highlights the critical need to isolate and characterize these unidentified host proteins which are directly or indirectly associated with vascular-dependent accumulation of viruses. For those whose function is to support movement, it will be interesting to determine their importance for trafficking host macromolecules through PD; for a description of one host protein associated with PD and a discussion of the subject see Epel et al. (1996).

It is becoming apparent that certain cell types within the bundle sheath (BS) are more easily infected than others. McCauley and Evert (1989) noted that particles of an unidentified virus often were present in VPCs but not CCs of minor veins of mature leaves from *Solanum tuberosum*. In tobacco, a similar observation was made for TMV (X. S. Ding et al. 1995). In that study, TMV was observed in only ~10–30% of the CCs compared with 60–90% of the VPCs at 3 dpi. Even at 7 dpi, a very late period post-inoculation, the percentage of CCs infected was no more than 30%.

Both *S. tuberosum* and *N. tabacum* contain minor veins having smooth-walled CCs. There are three types of minor veins observed within dicotyledons, each characterized by CCs that vary greatly in their structure and in the number of plasmodesmal connections between them and VPCs or BSCs (see Fig. 1 for examples of minor veins and Sect. 2.b for further discussion). The number of PD connecting the CCs with other cells can vary more than 1000-fold between species with different minor vein types and in some species the SE/CC complex is nearly symplasmically isolated. The ability of viruses to rapidly infect shoot apices of plants representing all three CC types indicates that either viruses require only a few functional PD between CCs and other cells in the inoculated leaf to allow systemic infection or that alternative routes for vascular invasion exist.

Ding et al. (1997) have recently completed a survey of various virus/host combinations to characterize the infection pattern exhibited by each in minor veins from inoculated leaves of dicotyledons. They determined that in no instance was a CC infected without a VPC also being infected, and very often VPCs alone were infected. They also determined that during the time of systemic symptom appearance on *Phaseolus vulgaris* and *Pisum sativum*, both infected with sunn-hemp mosaic tobamovirus, there were no smooth-walled CCs or TCs, respectively, that contained viral CP. It was concluded that, for the tobamoviruses and potyviruses studied, movement into VP cells occurred preferentially and likely prior to CCs. The data also suggest that viruses may not need to infect CCs prior to systemic movement. If virus moves through PD connecting VPCs with SEs, this could have an impact on models describing phloem loading mechanisms for carbohydrates if substantial quantities of sugars flow with virus into the SEs. Problematic for the postulate is the observation that PD between VPCs and SEs are often rare and in some cases have not been observed (e.g. McCauley and Evert 1989; Beebe and Evert 1992). Further research is necessary to determine the role of PD between VP cells and SEs in virus spread.

The above model for virus transport makes the assumption the virus must pass through BSCs and then through VPCs and/or CCs to reach the SEs of minor veins. Ding et al. (1997) also discuss the possibility that viruses gain entrance to SEs through termini of minor veins (i.e. vein endings). In *Beta vulgaris* and *Syringa* leaves, MCs or BSCs abutting SEs were observed (Esau 1967, 1977). Therefore, in this and other species having similar vein structures viruses would not have to pass through VPCs or CCs to gain access to SEs. There is one report where an MC at the terminus of a minor vein was infected. In a potato plant, secondarily-infected with potato leafroll virus (PLRV), an MC at the terminus of a minor vein was heavily infected (van den Heuvel et al. 1995). It is now important to determine if a virus can enter vascular tissue through this route as well as potentially exit from it.

In many plant species, however, SEs do not extend to the ends of minor veins, and in some species few veins with termini exist (Horner et al. 1994). In these species, virus would likely have to access SEs through VPCs or CCs since these cells generally encircle the SEs without gaps (e.g. Ding et al. 1997).

Another potential access point for viruses to the vasculature is at vein branches which may have gaps between the VPCs and CCs thereby allowing MCs or BSCs to abut SEs. Minor veins are not always regular in appearance, with SEs sometimes being in direct contact with BSCs [see Fig. 1 (left panel)]. Micrographs of some minor veins show gaps between BSCs (Turgeon and Hepler 1989) while others do not at intersecting veins (e.g. Mauseth 1988). A systematic literature search and research plan is necessary to determine whether gaps do exist and can allow virus entry through cells other than the VPCs or CCs.

Identification of the vein orders in inoculated leaves utilized by viruses for systemic spread and the pathway through the cells of these veins will be among the most technically demanding of all the studies on vascular transport of viruses. To undertake these studies, the inoculation site and spread of viruses from the site must be limited such that only a particular vein order is accessible for infection. The path the virus takes must be marked in some manner, thus allowing the researcher to follow cell-to-cell movement. One possibility to mark the path is to fuse a reporter gene (e.g. GFP) with a viral gene. The reporter gene product must be visualized after taking a section of leaf tissue since it is unlikely that all the cells in the region of the vein can be visualized from one plane with a confocal microscope. If viruses gain entry into SEs at the vein endings or through MCs or BSCs inserted into gaps between vascular cells, these sites would need to be fairly regular and accessible regardless of plant species since the rate of virus spread is consistently rapid (5–7 dpi) in all plants. Regardless of the exact location of entry, the influence of such macromolecular "leakage" on phloem loading or host macromolecular transport would require study.

As a final note in this section, Gamalei et al. (1994) have determined that assimilate transport is affected in symplasmic loaders by dropping the temperature from  $> 20$  to  $10^{\circ}\text{C}$ . A contraction of the endoplasmic reticulum (ER) labyrinth in intermediary cells (ICs) was one of the major effects of this temperature shift and the authors discuss the potential that this contraction affects transport of photosynthate through the PD of ICs. It would be interesting to determine the effect of such a temperature shift on virus transport for these symplasmic loaders.

## b) Entry to Sieve Elements

Although the invasion pathway through the vein cells in an inoculated leaf is a mystery, there is no question that SEs are the final destination for those viruses that are transported through the phloem. Through the production of mutant viruses, it has been shown that certain viral proteins are critical for spread of virus either into or through the sieve tubes for accumulation in distant locations. The CP is often essential for this phloem-dependent accumulation. A sampling of viruses that require a CP or a specific form of CP for efficient phloem-dependent accumulation, but not cell-to-cell spread, is shown in Table 1. For some viruses, such as CMV and tobacco etch virus (TEV), the situation is complicated in that only a portion of the CP is required for phloem-dependent accumulation while other portions of the proteins are required for cell-to-cell movement (Suzuki et al. 1991; Dolja et al. 1994; Table 2). Whether only portions of the CP are required for cell-to-cell or vascular-dependent movement of other viruses listed in Table 2 will be interesting to determine. Recently, Schneider et al. (1997) obtained results suggesting that the carboxy terminal two-thirds of the CCMV CP was sufficient to allow systemic movement of this virus.

For TMV, TRV and RCNMV it has been suggested that the virion (i.e. the encapsidated virus) is necessary for phloem-dependent accumulation (Sanger 1969; Oxelfelt 1975; Saito et al. 1990; Vaewhongs and Lommel 1995). The studies on TMV give the strongest evidence for this. Oxelfelt (1975) determined that a mutant TMV, which encapsidates only at a low temperature, accumulates systemically only at a low temperature. Saito et al. (1990) mutated the RNA sequence that serves as the origin for encapsidation for TMV without altering the amino acid sequence of the encoded MP. This mutant virus moved poorly in *N. tabacum*, thus indicating that encapsidation was important for efficient phloem-dependent accumulation. However, others have speculated that the role of the CP for TMV and PVX is to interact with the viral RNA, but not to form a virion (Dorokhov et al. 1984). Also, Dolja et al. (1994) noted that TEV may move without a CP; however, the potential that they were unable to detect a minute quantity of an encapsidated form of their mutant virus in CP-expressing plants was noted. To further study the location where the CP functions in vascular-dependent accumulation, it would be worthwhile to express the CP behind a CC-specific promoter. The CP expressed from the transgene could complement virus movement to the shoot only if the virus was capable of cell-to-cell movement into CCs. TMV mutants lacking functional CPs can invade VPCs and, in some cases, CCs (Ding et al. 1996). If such a virus could move to the shoot apex by complementation with the transgene-expressed CP, the location where the CP functioned would be identified.



**Table 1.** Virus/host combinations that require a CP or a specific form of CP for efficient phloem-dependent accumulation, but not cell-to-cell spread

Virus	Host	Reference
TMV	<i>N. tabacum</i>	Siegel et al. (1962), Dawson et al. (1988), Takamatsu et al. (1987)
CaMV	<i>N. bigelovii</i> and <i>D. stramonium</i>	Qiu and Schoelz (1992)
TYMV	<i>B. rapa</i>	Bransom et al. (1995)
TAV	<i>C. sativus</i>	Taliansky and García-Arenal (1995)
BMV <sup>a</sup>	<i>C. hybridum</i>	Flasinski et al. (1995)
BNYVV	<i>S. oleracea</i>	Quillet et al. (1989)
TRV	<i>N. tabacum</i>	Sänger (1969), Hamilton and Baulcombe (1989)
BGMV	<i>N. benthamiana</i>	Pooma et al. (1996)
TGMV	<i>D. stramonium</i> and <i>N. tabacum</i>	Jeffrey et al. (1996), Pooma et al. (1996)
CyRSV	<i>N. clevelandii</i> and <i>N. benthamiana</i>	Dalmay et al. (1992)
CCMV <sup>b</sup>	<i>V. unguiculata</i>	Allison et al. (1990)
TEV $\Delta$ N or $\Delta$ C <sup>c</sup>	<i>N. tabacum</i>	Dolja et al. (1994, 1995)
CMV + $\Delta$ SN <sup>d</sup>	<i>V. unguiculata</i> and <i>N. tabacum</i>	Suzuki et al. (1991)
RYMV <sup>e</sup>	<i>O. sativa</i>	Brugidou et al. (1995)
RCNMV	<i>N. benthamiana</i>	Vaewhongs and Lommel (1995)
BWYV <sup>f</sup>	<i>N. clevelandii</i>	Ziegler-Graff et al. (1996)
SBMV	<i>P. vulgaris</i> in presence of SHMV	Fuentes and Hamilton (1993)
TCV <sup>g</sup>	<i>B. campestris</i>	Heaton et al. (1991) Hacker et al. (1992)

TMV, tobacco mosaic virus; CaMV, cauliflower mosaic virus; TYMV, turnip yellow mosaic virus; TAV, tomato aspermy virus; BMV, brome mosaic virus; BNYVV, beet necrotic yellow vein virus; TCV, turnip crinkle virus; TRV, tobacco rattle virus; BGMV, bean golden mosaic virus; TGMV, tomato golden mosaic virus; CyRSV, cymbidium ringspot virus; CCMV, cowpea chlorotic mottle virus; TEV, tobacco etch virus; CMV, cucumber mosaic virus; RYMV, rice yellow mottle virus; RCNMV, red clover necrotic mosaic virus; BWYV, beet western yellows virus; SBMV, southern bean mosaic virus; SHMV, sunn-hemp mosaic virus.

<sup>a</sup> This has been questioned recently by Rao and colleagues (Rao and Grantham 1996; Schmitz and Rao 1996 and references therein) who, along with others (Sacher and Ahlquist 1989), have shown that CP may be essential for cell-to-cell spread in *C. quinoa* and *H. vulgare*.

<sup>b</sup> CP may influence cell-to-cell movement since virus accumulation in inoculated leaves, representing cell-to-cell movement, was < 1:100 of normal.

<sup>c</sup> TEV missing either N terminal [amino acid sequence (aas) 1–29] or C terminal (aas 246–262) portions of CP, i.e.  $\Delta$ N or  $\Delta$ C.

<sup>d</sup> CMV with a deletion of 26 amino acids near N terminus of CP (i.e. +  $\Delta$ SN).

<sup>e</sup> CP likely influences cell-to-cell movement since virus accumulation in inoculated leaves, representing cell-to-cell movement, was well < 1:100 of normal.

<sup>f</sup> CP may influence cell-to-cell movement since virus accumulation in agro-inoculated tissue was low.

<sup>g</sup> CP may influence cell-to-cell movement since virus accumulation in inoculated leaves, representing cell-to-cell movement, was well below normal (Laakso and Heaton, 1993).

**Table 2.** Virus/host combinations where CP is required for cell-to-cell spread

Virus	Host	Reference
PVX	<i>N. tabacum</i> and <i>N. clevelandii</i>	Chapman et al. (1992a,b)
WCLMV	<i>N. clevelandii</i>	Forster et al. (1992)
TEV	<i>N. tabacum</i>	Dolja et al. (1995)
CPMV	<i>V. Unguiculata</i>	Wellink and van Kammen (1989)
AIMV <sup>a</sup>	<i>N. tabacum</i>	van der Kuyl et al. (1991)
CMV	<i>V. Unguiculata</i>	Suzuki et al. (1991)
	<i>N. tabacum</i>	Boccard and Baulcombe (1993) <sup>b</sup>
MSV	<i>Z. mays</i>	Boulton et al. (1989), Lazarowitz et al. (1989)
BCTV	<i>N. benthamiana</i> and <i>B. vulgaris</i>	Briddon et al. (1989)
TLCV	<i>L. esculentum</i>	Rigden et al. (1993)

PVX, potato virus X; WCLMV, white clover mosaic virus; TEV, tobacco etch virus; CPMV, cowpea mosaic virus; AIMV, alfalfa mosaic virus; CMV, cucumber mosaic virus; MSV, maize streak virus; BCTV, beet curly top virus; TLCV, tomato leaf curl virus.

<sup>a</sup> CP requirement may be for RNA synthesis and not cell-to-cell movement, or necessary for both.

<sup>b</sup> Data not shown.

For viruses that are transmissible by aphids, it is reasonable to think that movement in the sieve tubes is as a virion, since feeding aphids would accumulate the virus from SEs and, at least for potyviruses, there is a requirement for a sequence within the CP for aphid transmission (Atreya et al. 1990, 1991). However, the aphid may acquire the encapsidated forms from the VPCs or CCs while probing for entry into the SE with the stylet. Thus, the viral transit form is not known for certain for such viruses.

Determining the form in which these viruses move is essential for our understanding of how viruses interact with host factors during entry, transit and exit from the sieve tubes or xylem vessels. That virus transit forms do interact with host factors was shown through studies with chimeric viruses (TMV containing the CP of odontoglossum ringspot tobamovirus and cymbidium ringspot tombusvirus containing the CP of artichoke mottle crinkle tombusvirus; Burgyán et al. 1993; Hilf and Dawson 1993) or viral genomic sequences from two parental strains (CMV with the CP of tomato aspermy cucumovirus; Taliansky and García-Arenal 1995). It was determined in these studies that host factors, separate from those important for cell-to-cell spread, interact with CPs or the capsids from some of these viruses to allow phloem-dependent accumulation (Table 3).

**Table 3.** Viruses shown to require specific host factor – CP interactions for phloem-dependent accumulation

Virus	Host	Reference
TMV/ORSV*	<i>N. tabacum</i>	Hilf and Dawson (1993)
CMV/TAV	<i>C. sativus</i>	Taliansky and García-Arenal (1995)
CyRSV/AMCV	<i>N. clevelandii</i>	Burgyán et al. (1993)

TMV, tobacco mosaic virus; ORSV, odontoglossum ringspot virus; CMV, cucumber mosaic virus; TAV, tomato aspermy virus; CyRSV, cymbidium ringspot virus; AMCV, artichoke mottle crinkle virus.

\* Virus that moves systematically in host/source virus of CP that does not allow phloem-dependent accumulation and which replaced CP in virus capable of systemic infection.

Recently, a technical breakthrough has been published that will aid in the study of virus transit forms and their interactions. PVX was modified by fusing the gene encoding the fluorescent protein, GFP, with the gene encoding the CP, leading to an encapsidated, green fluorescing virus (Oparka et al. 1995b; Santa Cruz et al. 1996). This virus moves systemically in a phloem-dependent manner and should aid in determining the form (i.e. encapsidated or unencapsidated) in which this virus moves, assuming sufficient quantities of the movement form exist to allow visualization by analysis of stem exudates, stylet exudates or live stem phloem cells in real time. Use of GFPs whose sequences have been modified to increase fluorescence (up to 150-fold) or alter the emission spectra should help in this visualization process (Cramer et al. 1996; Pang et al. 1996; Reichel et al. 1996). Even further improvements in the level or quality of fluorescence by GFP should appear in the future since the crystal structure of the protein has been solved to 1.9 Å (Yang et al. 1996).

The availability of green fluorescent virus and a viral construct where the gene encoding GFP has replaced the gene encoding a CP (i.e. virus ΔCP + GFP) may also help in identifying the location within the vein cells where the CP is essential for phloem-dependent accumulation. Recently, using immunocytochemical analyses to observe the accumulation of CP mutants of TMV, it was determined that the CP of this virus exerts its effect on phloem-dependent accumulation during entry into the SE/CC complex or beyond this point (Ding et al. 1996). This location is beyond the boundary, namely the BSC/VPC – SE/CC boundary, previously suggested to be the site of the CP effect (Ding et al. 1992; Lucas and Wolf 1993, see Sect. 4.a). Using the two GFP constructs (i.e. the green virus and the ΔCP + GFP virus), researchers may be able to identify the boundary where CP becomes necessary for vascular transport of those viruses requiring the CP for this function.

The involvement of MPs in entry of viruses into SEs has been difficult to study due to the requirement for the MP in cell-to-cell movement of the viruses and because, to date, injection of viral proteins into CCs or SEs has not been achieved. In one study, the suggestion was made that MPs could influence phloem-dependent accumulation (Fenczik et al. 1995), but this possibility is confounded by the fact that viruses in this study having slow phloem-dependent accumulation also have reduced cell-to-cell movement. This reduced cell-to-cell movement may have been the result of a nonnecrotic defence mounted by the host (see Goulden and Baulcombe 1993; Köhm et al. 1993; Scholthof et al. 1993; De Jong and Ahlquist 1995; Fenczik et al. 1995; Mise and Ahlquist 1995; Bao et al. 1996; Derrick et al. 1997 for discussions of this phenomenon). At some point it may be possible to inject MP-defective viruses into CCs and determine whether MP expressed behind CC-specific promoters complements virus movement to the shoot apex. Such complementation experiments are similar in concept to those outlined earlier in this section (Sect. 4.b) for viruses lacking CPs, and will help determine if and where the MPs are necessary for phloem transport.

Viral proteins other than CPs or MPs or their encoding RNAs have also been shown to affect phloem-dependent accumulation of viruses directly or indirectly (e.g. Wintermantel et al. 1993; Table 4). In some studies, the cause of the diminished phloem-dependent accumulation is likely due to decreased production of infectious virus in the inoculated leaves (e.g. Lewandowski and Dawson 1993) or possibly to a lack of a sufficient quantity of MP (Watanabe et al. 1987; De Jong and Ahlquist 1995). However, in other studies, the lack of phloem-dependent accumulation cannot easily be attributed to a lack of infectious virus or viral MP since both accumulated to substantial levels in inoculated leaves (Gal-On et al. 1994; Derrick et al. 1997). For TMV, the 126/183-kDa proteins and not the encoding RNA are the cause of differential symptoms and the likely cause of the reduced phloem-dependent accumulation between an attenuated masked (M) and severe (U1) strain of the virus (Bao et al. 1996; Derrick et al. 1997).

In only two studies has the location where nonstructural viral proteins exert their effect been addressed. In these studies, viruses containing mutations in the protein sequence were studied for their movement phenotypes compared with the wild type virus. As mentioned previously (see Sect. 4.a), sequences in the 126/183-kDa protein of TMV likely regulate virus accumulation within VPCs and CCs of fifth-order or similar size veins but not in the surrounding BSCs (X. S. Ding et al. 1995). Thus, the impact of this orf appears to be prior to entry into SEs and it will be important to separate this function from any function of the sequence for entry into SEs. The helper component-proteinase (HC-Pro) protein of TEV was shown to accumulate in vein cells (probably both VPCs and CCs), thus leading the authors to speculate that this

**Table 4.** Sampling of non-structural proteins, other than MP, from RNA viruses that affect phloem-dependent accumulation directly or indirectly

Non-structural Protein	Virus	Host	Reference
HC-Pro 126/183 kDa	TEV	<i>N. tabacum</i>	Cronin et al. (1995)
	TMV (strain M)	<i>N. tabacum</i>	Derrick et al. (1997), X. S. Ding et al. (1995)
126/183 kDa	TMV (strain V-36)	<i>N. tabacum</i>	Lewandowski and Dawson (1993)
126/183 kDa	TMV (strain L <sub>1</sub> ,A)	<i>N. tabacum</i>	Nishiguchi et al. (1985), Watanabe et al. (1987)
126/183 kDa and/or CP	SHMV	<i>N. tabacum</i>	Deom et al. (1994)
126/183 kDa and/or CP	RCMV/TMV (strain LSI)	<i>N. tabacum</i> expressing MP of TMV	Taliansky et al. (1992)
1a	CMV (strain Sny)	<i>C. pepo</i>	Roossinck and Palukaitis (1990), Gal-On et al. (1994)
1a	CMV	<i>N. tabacum</i>	Lakshman and Gonsalves (1985)
1a	BMV (strain M1)	<i>V. unguiculata</i>	De Jong and Ahlquist (1995)
1	CCMV (strain T)	<i>V. unguiculata</i>	Wyatt and Kuhn (1980)
αa	BSMV (strain ND18)	<i>A. sativa</i>	Weiland and Edwards (1994, 1996)
2	BMV (strain PT14)	<i>A. vulgare</i>	Traynor et al. (1991)
2	CMV (strain LsS)	<i>P. sativa</i>	Edwards et al. (1983)
2b	CMV (strain Q)	<i>C. sativus</i>	S. W. Ding et al. (1995)
p19	TBSV (strain pH5157)	<i>S. oleracea</i>	Scholthof et al. (1995)

HC-Pro, helper component-proteinase; TEV, tobacco etch virus; TMV, tobacco mosaic virus; SHMV, sunn-hemp mosaic virus; RCMV, red clover mottle virus; CP, coat protein; CMV, cucumber mosaic virus; BMV, brome mosaic virus; CCMV, cowpea chlorotic mottle virus; BSMV, barley stripe mosaic virus; TBSV, tomato bushy stunt virus.

protein functions to allow entry into or exit from SEs of fifth-order veins (Cronin et al. 1995). In all these studies, the impact of a virus-induced host resistance response must be closely monitored (for a likely example of this see the impact of RNA2 of CMV strains B and LsS on infection of *Pisum sativum* or *Vigna unguiculata*; Edwards et al. 1983).

There has been one report where virus spread into stem SEs was followed (de Zoeten and Gaard 1983). In this study, fully expanded leaves of *P. sativum* were inoculated with pea enation mosaic virus and stem sections directly above the node of the inoculated leaf were analysed. By 4 dpi, vesicles appeared in the mature SEs and their appearance coincided with that of double-stranded (ds) RNA. The authors did not determine whether this dsRNA was of viral origin, but previous studies had shown that vesicles containing RNA-dependent RNA polymerase and

virus-specific dsRNA were produced in cells (Powell and de Zoeten 1977; Powell et al. 1977 and references therein). Therefore, for this virus there may be a requirement for replication-associated proteins and not a CP for phloem-dependent accumulation.

The impact of host factors on entry of virus into SEs is poorly studied. Schaad and Carrington (1996) have determined that a resistance gene in *N. tabacum* inhibits vascular-dependent accumulation of TEV. The trait was recessive and multigenic and virus was able to accumulate in VPCs and possibly CCs of inoculated leaves. It was concluded that this resistance functioned somewhere at or beyond the SE/CC interface to prevent systemic accumulation.

## 5. Transport of Virus Through Sieve or Tracheary Elements

### a) Historical

Rapid (centimeters per hour) movement of plant viruses from inoculated leaves to other areas of the plant was first reported in the 1920s and 1930s (e.g. see Caldwell 1930; Holmes 1930; Samuel 1934; Bennett 1940 and references within these articles). Through girdling experiments, where living cells in petioles or stems were killed, it was shown that many viruses cannot move through dead tissue and thus require the presence of living tissue for systemic infection (e.g. Caldwell 1930 and references therein). More recently, this approach has been used to show that CaMV moves through the phloem (Leisner et al. 1992). For viruses moving through living tissue, there was some initial controversy regarding whether movement occurred through ground tissue (Caldwell 1934) or vascular tissue (Holmes 1930, 1932). Samuel (1934), using indirect evidence, explicitly stated that TMV moves through vascular tissue. Bennett (1956) reviews this early literature in detail.

In the late 1960s and early 1970s, evidence became abundant that virus particles, believed to travel through phloem tissue, accumulate in vein cells (e.g. Esau and Cronshaw 1967; Esau et al. 1967; Esau 1968; Hoefert et al. 1970; Esau and Hoefert 1971 and references within these articles). In these studies, virus was observed not only in VPCs and CCs, but also in SEs. For some host/virus combinations, virus was also observed in xylem vessels (Esau and Cronshaw 1967; Esau and Hoefert 1971). Esau and Cronshaw (1967) noted that the presence of virions in SEs may indicate that the virus moves as a virion. However, these studies did not determine the time of appearance of virus in these cells relative to the development of symptoms in phloem-accessed tissue. Therefore, it is not possible to determine whether virus in these cells represents a movement form or simply accumulation of virus after movement occurred.

Through girdling experiments, some viruses have been shown to move through dead tissue, thus implicating xylem tissue in their movement (Schneider and Worley 1959; Gergerich and Scott 1988; Urban et al. 1989). Interestingly, the accumulation of lettuce necrotic yellows virus and beet necrotic yellow vein virus in vessel elements was suggested to occur before their differentiation (Chambers and Francki 1966; Dubois et al. 1994). Esau and Cronshaw (1967) observed TMV in immature tracheary elements and immature SEs. These findings point out another confounding aspect in interpreting results from both xylem-dependent and phloem-dependent movement studies, namely that the developmental status of the vein cells at the time of virus invasion may play an important role in vascular-dependent infection. Therefore, when observing the presence of virus in xylem vessels or phloem SEs the maturity of the cells in the tissue section must be taken into account, whether it be in inoculated leaves or in systemically infected leaves. Infection of immature vessels or elements may allow entrance into or exit from the transport system in some instances (also see discussion in Sect. 2.a).

#### b) Grafting and Transport Experiments

In regard to the need for viruses to replicate while moving through SEs, several grafting studies have addressed this issue. In early work, Baur (quoted in Caldwell 1930) grafted an immune stem of one plant onto a susceptible plant and observed movement through it. However, Blakeslee (1921) did not obtain a viral disease of *Datura* by passage through a resistant petunia stock under an infected scion. White and Sugars (1996) determined that stems of tobacco plants expressing the N-gene, which localize TMV infections through the necrotic hypersensitive response, when used as interstocks allowed TMV to pass through and establish infection in tobacco lacking the N-gene. Although results from this study might be taken to suggest that TMV does not have to replicate as it passes through the phloem, it is possible that replication and movement occurs more quickly than the resistance response can prevent it (White and Sugars 1996).

As noted previously, for many viruses their CP is necessary for long-distance spread (see previous section and Table 1); however, the cellular location(s) where it functions in this role is unknown. The importance of the CP during viral transport through SEs may be to stabilize the virus from nuclease attack while in transit (discussed by Oxelfelt 1970). However, it has never been shown that RNases are present in SEs and some viruses apparently can move fairly quickly through the vascular tissue without a CP (Table 5). Also, transport of potato spindle tuber viroid (a plant RNA pathogen that does not encode a protein) has been shown to occur in a phloem-dependent manner, i.e. by transport to strong sink

**Table 5.** Virus/host combinations that do not require CP for systemic invasion

Virus	Host	Reference
TBSV	<i>N. benthamiana</i> and <i>N. clevelandii</i>	Scholthof et al. (1993)
BSMV	<i>N. benthamiana</i>	Petty and Jackson (1990)
RCNMV	<i>N. benthamiana</i> at reduced temperatures	Xiong et al. (1993)
CNV	<i>N. clevelandii</i>	Rochon et al. (1991)
TGMV	<i>N. benthamiana</i>	Brough et al. (1988), Gardiner et al. (1988)
BGMV	<i>P. vulgaris</i>	Azzam et al. (1994)
TLCV	<i>L. esculentum</i>	Padidam et al. (1995)
SqLCV	<i>C. maxima</i>	Ingham et al. (1995)
ACMV	<i>N. benthamiana</i>	Etessami et al. (1989)

TBSV, tomato bushy stunt virus; BSMV, barley stripe mosaic virus; RCNMV, red clover necrotic mosaic virus; CNV, cucumber necrosis virus; TGMV, tomato golden mosaic virus; BGMV, bean golden mosaic virus; TLCV, tomato leaf curl virus; SqLCV, squash leaf curl virus; ACMV, African cassava mosaic virus.

tissue (Palukaitis 1987). Thus, for this hypothesis to be viable, host proteins or viral proteins other than the CP would have to protect the viral genome, and nucleases would have to be present in the SEs. Another possible explanation for a requirement of the CP or some other viral or host factors in virus transport is to protect or stabilize the viral RNA or virus from the high pH (~7.5–8.0) found in SEs. Such a pH may disrupt tertiary RNA structures necessary for systemic infection (e.g. see Mundry et al. 1991 for the effect of pH 7.2 on the uncoating of the TMV capsid).

CP expressed in transgenic plants has been shown to limit the vascular-dependent accumulation of both TMV and PLRV (Wisniewski et al. 1990; Derrick and Barker 1992, 1997). TMV accumulated to similar levels in leaves of CP-expressing transgenic and non-transgenic plants inoculated with TMV RNA, but systemic accumulation was delayed by approximately 1 day in the transgenic plants. For PLRV, the lack of systemic virus accumulation was correlated with a lack of virus present in the abaxial phloem. The mechanism of this inhibited virus accumulation is unknown. Also, the specific effect on PLRV accumulation in abaxial and not adaxial phloem indicates another area requiring study: the influence of abaxial and adaxial phloem on virus movement.

In regard to the MP, Gera et al. (1995) determined that a mutant TMV lacking the MP gene (TMV MP<sup>-</sup>) can rapidly move through a nontransgenic interstock region of a host lacking the MP gene (MP<sup>-</sup>). Thus, this virus does not need the MP for rapid vascular spread. Since it is unlikely that viruses can replicate in SEs, these results indicate that either the



virus does not need to replicate along the way or, if the virus must replicate along the way, it can move into CCs from SEs in the absence of MP for replication. These researchers also observed that an infectious form of the TMV MP- virus was present in MP- tissue after phloem-dependent translocation from MP+ tissue. They could not detect this virus in MP- tissue by sap infectivity assays. Again, this virus could have accumulated as (1) a movement form or (2) a replication product in associated CCs, if MP is not needed to enter these cells. The former possibility seems more likely, since replicated virus would likely have accumulated to a level that was detectable by sap infectivity assays.

Dawson and Schlegel (1973) designed a system (i.e. the magic box) where mature portions of a plant can be placed in a controlled environment chamber for growth at ~29 °C while the shoot apical regions were placed at ~4 °C (TMV) or ~11 °C (CCMV) to inhibit virus replication in this tissue. Viruses, either TMV or CCMV, inoculated on mature leaves replicated and an infectious form moved to the shoot apices. These authors also found that leaves from shoot apices, detached at the time of transfer to 25 °C, accumulated virus at the same time as leaves transferred to 25 °C and left on the plants. Therefore, the infectious virus was present at the time of transfer. However, they could not detect infectious virus from extracted sap or isolated RNA. Thus, the movement form was unidentified.

The importance of replicase-associated proteins for SE transport beyond their role in replication is not understood. As mentioned previously (see Sect. 4.b), it is possible that the RNA-dependent RNA polymerase of pea enation virus is present in SEs (de Zoeten and Gaard 1983).

Host factors specifically involved in transport of viruses through transit phloem have not been identified. In fact, it has been hypothesized that transport through SEs is passive (see Atabekov and Dorokhov 1984). However, results from some reports suggest the presence of a host factor or factors that are active during transport. Through a double grafting experiment, a cultivar of potato was identified that slowed the rate of spread of potato leafroll luteovirus (PLRV) through its stem compared with five other cultivars (Wilson and Jones 1992). This inhibited movement was virus-specific, since PVX was not inhibited in movement. In similar studies, stem sections of resistant lines of *Lycopersicon peruvianum* inhibited transport of PLRV and tomato yellow top virus (Hassan and Thomas 1988). A requirement for an active host factor implies there would be an active viral factor with which to interact. Fenczik et al. (1995) speculated that the MP of TMV could affect the movement of virus into or out of SEs in the transit phloem. The basis of this suggestion was the observation that long-distance spread of a TMV mutant lacking an MP gene only occurred when stem tissue produced MP. Arce-Johnson et al. (1997) have recently expanded on this finding.

They found that only MP expressed in vascular tissue of an intergraft could complement the movement of a TMV mutant lacking the MP in the time frame studied. They also found that wild type virus could not move through an intergraft expressing the 54-kDa protein from TMV. This protein is included in the carboxy terminus of the 183-kDa protein sequence and has been shown to give near immunity to plant virus infections (Carr and Zaitlin 1991). Although results with the transgenic MP expressed in the intergraft could be interpreted as simply allowing virus to exit vascular tissue, replicate and spread cell-to-cell to the scion where it could reenter the SEs, the results from the transgenic 54-kDa protein intergraft suggest that replication is essential or that there is an active host defence response that recognizes the movement form and functions in SEs.

Recent results with PLRV shed some additional light on the impact of viral genes on sieve tube transport. PLRV is translocated through the SEs and multiplies predominantly in CCs (see Derrick and Barker 1997 and references therein). Recently, the 17-kDa movement protein was further characterized and found localized to the plasmodesmal area (Tacke et al. 1993; Schmitz 1995). The protein is present in the sieve tubes in its phosphorylated form. The phosphorylation is executed by a membrane-associated protein kinase (Sokolova et al. 1997). The results suggest that this protein functions to aid PLRV movement between the SE and CC; however, whether it functions for both entrance into and exit from the SE is not clear. Perhaps the MP is not required for spread into CCs and spread into these cells is necessary for systemic spread. Further research is necessary to relate these results with those of Gera et al. (1995; discussed previously in this section) and determine if and, if so, what host and viral factors impact virus movement through transit phloem.

Transport of viruses and viroids in a phloem-dependent manner could be a unique event, wherein the pathogen has evolved to create its own transport network independent of that for host macromolecules, or it could be construed as a normal event, wherein the virus has evolved to utilize an existing system in the host. Host proteins have been observed in SEs (Eschrich et al. 1971 and see van Bel and Kempers 1997 for review), thus lending support to the theory that a system exists for host protein transport. In later studies, ubiquitin,  $\beta$ -amylase and protein chaperones were identified as part of the protein complement identified within SEs (Schobert et al. 1995; Wang et al. 1995). Antibodies against P-proteins from *Streptanthus tortuosus* callus labelled only SEs from *Arabidopsis* leaf sections (Tóth et al. 1994). Interestingly, thioredoxin has also been found in sieve tube exudates (Ishiwatari et al. 1995). Thioredoxin was shown to increase the SELs of PD and potentiate its own cell-to-cell movement (cited in Lucas 1995). The normal presence of host proteins in SEs suggests that viruses have taken advantage of an existing system, just as it has been suggested they have done for cell-to-

cell movement (see Carrington et al. 1996; Gilbertson and Lucas 1996). Therefore, as in studies of cell-to-cell movement of macromolecules, viruses should serve as a model for macromolecules in studies of protein trafficking through vascular tissue.

Van Bel and van Rijen (1994) determined that the SE/CC complex of stems was increasingly electrically isolated from other cells (e.g. VPCs) as they matured from the cambial stage onward (see discussion in Sect. 2.e). It is interesting that nonvascular stem cells of tobacco eventually become infected by TMV, within a time period restricting the source of viral inoculum in the stem to the phloem (Nelson et al. 1993; Derrick et al. 1997). Also, van Lent and Verduin (1987) utilizing the magic box system of Dawson and Schlegel (1973), detected cowpea chlorotic mottle virus CP in phloem parenchyma cells from systemically inoculated petioles of *V. unguiculata* by 6-h post-transfer from 10 to 25 °C, and in phloem, BSCs and cortex cells by 24-h post-transfer. It has been suggested that a few plasmodesmal connections between the SE/CC complex and surrounding cells might account for a symplasmic mode of photosynthate unloading in stem cells. Future studies will determine whether viruses utilize such a route or make their own. Patrick and Offler (1996) demonstrated that when the prevailing source/sink ratio favours net assimilate storage in stems, unloading may be symplasmic. Since viruses likely move symplasmically, it would be interesting to see if stem invasiveness by viruses is positively correlated with an excess of photosynthate in sink tissue.

## 6. Exit from Vascular Tissue in Leaves

Once virus reaches the sink tissue of the plant by movement through the phloem, it must exit the vasculature and establish infection. Hatta and Matthews (1974) studied the accumulation of turnip yellow mosaic tymovirus (TYMV) in small systematically infected Chinese cabbage (*Brassica pekinensis*) leaves at 5–6 dpi. They determined that the first cells to be visibly infected were adjacent to vein cells. The portions of the veins observed contained 30 cells in transverse section and thus were not the highest order (i.e. smallest) veins. They did not comment on or show data supporting or denying the potential that virus can exit out of smaller veins. They did determine that virus appeared first in cells adjacent to the SE/CC complex and VPCs and not from xylem-associated cells. Unfortunately, the cells within the transport pathway out from the veins were not identified, and therefore, the ability of virus to infect CCs or VPCs directly from SEs was not addressed. Hoefert et al. (1988) studied the accumulation of lettuce infectious yellows virus in systemically infected leaves of *Lactuca sativum* over time. They identified virus-induced vesicles and virus particles in TCs and VPCs at 5 days post-

inoculation. The developmental stage of the leaves and the vein orders analyzed were not fully described.

Recently, Valkonen and Somersalo (1996) followed the accumulation and movement of TEV in resistant and susceptible hosts and grafts between them. Using an engineered TEV expressing GUS they determined that the virus accumulated in leaves of a tobacco rootstock but could not infect leaves of a scion from *Solanum brevidens*, a resistant host to TEV. Although no detectable virus infection was established through vascular delivery of the virus, neither the completeness of the restriction (i.e. the extent of restriction of virus spread around the SE) nor the cell type in which the inhibition occurred was determined.

For TMV, a mutant lacking the MP moved via the phloem into tissue not expressing the MP and accumulated an infectious form of the virus there (Gera et al. 1995). However, neither the cellular location of the infectious form in the vein was identified nor was it determined whether the virus replicated upon its arrival in the scion. X. S. Ding et al. (1995) determined that the percentage of CCs and VPCs infected by TMV in minor veins of systemically infected leaves was nearly equal (~50 versus 60%). This was unlike the infection percentages of these cells in inoculated leaves (~90% of VPCs and 10–30% of CCs infected). The authors speculated that movement into CCs from SEs was freer because PD between these cells are present in abundance (Beebe and Evert 1992; Gamalei et al. 1994; Grusak et al. 1996) and the SELs between these cells are larger than at other cell interfaces (Kempers et al. 1993; Kempers and van Bel 1997). The infection of the VPCs in this study may be due to the greater number of PD between CCs and VPCs in immature tissue (see X. S. Ding et al. 1995 for discussion). Unfortunately, a vectorial flow out from SEs was not identified in this study, and thus another possibility is this tissue may have been infected due to a wave of virus moving cell-to-cell from an adjacent vein. In such a scenario, virus could have infected both immature VPCs and CCs due to the high number of PD present in the immature tissue. This scenario seems even more likely since it has recently been shown that PVX exits exclusively out of third-order and larger veins and not fifth-order veins in *Nicotiana benthamiana* (Roberts et al. 1997). So, as pointed out previously (see Sect. 5.a), it is extremely important to consider the developmental stage of the cells under study before making final conclusions about the meaning of data for transport of viruses into or out of the phloem.

For phloem-limited RNA viruses, spread from SEs to other cells in systemically infected tissue appears to be limited to CCs and, rarely but interestingly, to a few VPCs and MCs (D'Arcy and de Zoeten 1979; Shepardson et al. 1980; Sanger et al. 1994; van den Heuvel et al. 1995). The near absence of these viruses in BSCs or MCs in spite of more plasmodesmal connections between either of these cells and VPCs versus VPCs and the SE/CC complex suggests that these viruses either cannot

replicate efficiently in BSCs or MCs or cannot enter them due to differences in PD between the BSCs and the VPCs and CCs. Since these viruses can replicate in protoplasts mostly derived from MCs (Barker and Harrison 1982; Sanger et al. 1994), it is more likely that the PD are modified to prevent movement. Further evidence for this comes from the observation that coinfection of potato potyvirus Y (PVY), a phloem nonlimited virus, with PLRV, a phloem-limited virus, results in a higher proportion of protoplasts (presumably from MCs) being infected on isolation from the double-infected *Nicotiana clevelandii* plants versus plants infected with only PLRV (Barker 1987a). Thus, PVY supplied a factor that released PLRV from phloem localization. The protein(s) involved in the release from phloem limitation is unknown. These results support the studies discussed earlier indicating that the PD between the BSCs and VPCs and/or CCs can regulate virus and photosynthate transport (see Sect. 4.a).

The phloem limitation of bean golden mosaic geminivirus (BGMV) is broken by coinfection with sunn-hemp mosaic tobacco virus (SHMV; formerly the legume strain of TMV; Carr and Kim 1983). As for the RNA virus coinfection work, the protein(s) responsible for breaking the phloem-limitation has not been identified. Recently, the distribution of another bipartite geminivirus, bean dwarf mosaic geminivirus (BDMV), within different tissue and cell types of systemically infected *Phaseolus vulgaris* and *N. benthamiana* was studied (Wang et al. 1996). Interestingly, BDMV was not limited to phloem in mature inoculated leaves of either *P. vulgaris* or *N. benthamiana*. It also was not phloem-limited in systemically infected immature leaves of *P. vulgaris* and *N. benthamiana* and in systemically infected mature leaves of *P. vulgaris* prior to 12 dpi. However, in immature leaves of *P. vulgaris* at 21 dpi, the virus was more noticeably restricted to the VPCs, CCs and BSCs. It appears from this work that the virus is able to overcome vascular restrictions as the tissue ages. Thus, for some viruses, escape from the vascular tissue appears dependent on developmentally regulated host factors in the shoot apex.

In a study with a monopartite geminivirus, maize streak geminivirus, on a monocot host, *Zea mays* (maize), it was shown that vascular limitation decreases during the development of the leaf (Lucy et al. 1996). The oldest leaf within the whorl had a nonvascular distribution of virus only in the mature (i.e. distal) portion of the leaf. The ability to move into nonphloem tissue corresponds with the maturation of metaphloem elements, which in turn corresponds with the end of cell division in the mature leaf (Lucy et al. 1996 and references therein). Although the ability of virus to escape vascular tissue may have a relationship to the number of PD present, it is not a perfect correlation in leaf tissue and appears to be negatively correlated with PD numbers in root tissue (Lucy et al. 1996). Van Bel and Oparka (1995) have commented that plasmodesmal frequencies do not always reflect the level of intercellular

communication between cells. Other factors, therefore, may limit infection in these instances. For example, the ability of the receiving cells to conduct photosynthesis (Lucy et al. 1996) or to developmentally regulate PD aperture may limit spread. The relationship of virus exit to photosynthate exit in sink tissue of maize will be interesting to determine since viruses almost certainly move in the symplasm and it has been suggested that photoassimilate must exit to the apoplasm in this plant (Evert and Russin 1993). It is worth noting that the study by Lucy et al. (1996) is the only study of virus movement in monocots. As noted in Section 2, monocots have a very different arrangement of vascular elements and phloem loading procedures from dicots (also see Kaneko et al. 1980; Chonan et al. 1984, 1985; van Bel et al. 1988). Considering the importance of monocots in agriculture, it will be important to study virus movement in this group.

For RNA viruses that are not limited to the phloem, similar statements have been made about the importance of tissue maturity for cell susceptibility to virus infection. Solberg and Bald (1962) noted that TMV shows a gradient of leaf infection wherein some leaves have the distal portion of the leaf infected and the basal portion virus free. Although it is possible that the virus was moving only during a defined period of time from the lower inoculated leaves and thus no inoculum was present for the tissue at the base of the leaf, it also possible that the young cells at the base were developmentally incompetent for virus infection. Solberg and Bald (1962) noted this in their paper: "It is suggested that tissue maturity gradients are associated with leaf susceptibility to virus invasion and multiplication in many plants".

Others also have discussed the fact that symptom formation is affected by leaf maturity at the time of infection (Holmes 1934; Zech 1952; Reid and Matthews 1966; Nilsson-Tillgren et al. 1969). In immature tobacco leaves, several complex developmental pathways are maturing during the period when TMV invades. These include completion of cell division, maturation of the vascular system and transition from a sink to a source tissue (Poethig and Sussex 1985a; Turgeon 1986; Ding et al. 1988, 1993; Volk et al. 1996; see Sect. 2.c for further discussion). The impact of these processes on the accumulation of plant viruses has not been fully studied.

In most studies where vascular-dependent virus accumulation has been altered (e.g. Gal-On et al. 1994; Dolja et al. 1994, 1995; Cronin et al. 1995; Derrick et al. 1997), it has yet to be proven that the inhibition is due solely to inhibition of virus movement. It is possible that the mutants in these studies simply cannot replicate in immature tissue. The inability to replicate could be passive, in that host factors for replication may be absent, or it could be active, wherein host factors inhibit replication. The latter case would be similar to the virus-induced host resistance which may occur in inoculated leaves to limit virus movement (see

Sect. 4.b and discussion of virus-induced host resistance in Goulden and Baulcombe 1993). Analysis of virus replication in mesophyll protoplasts from *mature* leaves is not sufficient to answer this concern. On the other hand, Nelson et al. (1993) determined that a delay in virus accumulation was apparent in the *mature* petioles and lower stem sections for the M strain of TMV, thus suggesting that at least a portion of its attenuated phenotype was accounted for by a delayed movement and not an inability to replicate in young systemic tissue. Also, Gal-On et al. (1994) determined that the attenuated strain of CMV which they studied was delayed in exit from the inoculated leaf by between 6 and 12 h post-inoculation. Both of these delays were transitory, however, and likely cannot entirely account for the visual phenotype. Evidence for this was obtained through the study of a mutant of the masked strain ( $M^{IC}m6$ ); this mutant induces severe symptoms but is similar to the M strain in exhibiting slow vascular-dependent accumulation (Shintaku et al. 1996; Derrick et al. 1997).

Some answers to the question of virus susceptibility of immature tissue could come from studies wherein immature leaves on a plant or protoplasts from young leaves are inoculated. In tobacco, however, such studies are difficult because immature leaves (~2 cm in midrib length) are recalcitrant to infection and are covered with a large number of leaf hair cells that prevent access to the laminar cells (Poethig and Sussex 1985b). Also, the number of laminar cells in these young leaves is very low (Poethig and Sussex 1985b), thus forcing one to produce protoplasts from an extraordinarily large amount of fresh tissue. It may be necessary to undertake this "brute-force" approach, however, to gain a definitive answer to this question.

For the DNA virus, CaMV, the pattern of virus accumulation in systemically infected tissue was similar to that of photoassimilate import, with one major difference: virus accumulation ended at an earlier stage of leaf development than did photoassimilate accumulation (Leisner et al. 1992). The authors speculate that either a higher threshold for systemic infection occurs compared with photoassimilate exit or the diameters of PD connecting the SE/CC complex with surrounding cells had slowly decreased, thereby excluding virus exit earlier than photoassimilate exit. These authors also observed nodular-shaped lesions spaced infrequently along major veins of systemically infected leaves. These results suggest, but do not prove, that virus exit is from predominantly third-order veins (i.e. major veins), these veins being the same veins used for photoassimilate exit (Turgeon 1986). They also suggest that exit of virus into the leaf lamina is not easily achieved.

The use of chimeric viruses containing GFP should be useful in unambiguously identifying the vein orders utilized by virus for exit from the vascular tissue. Roberts et al. (1997) have recently completed such a study following the exit of PVX and CFDA, representing photosynthate,

from *N. benthamiana* leaves in transition from sink to source. They determined that both virus and CFDA were released exclusively in sink tissue from third-order veins or larger and not fourth- or fifth-order veins. These are watershed findings and indicate quite clearly that plant viruses are utilizing the same pathway as photosynthate for invasion of sink tissue. One could surmise that host proteins being transported through the phloem would exit in a similar fashion. A mystery remains for monocots, however, in that photosynthate exit appears to be apoplasmic (Evert and Russin 1993).

## 7. Summary and Prospects

Remarkable progress has been made in understanding how viruses traffic into, through and out of the vasculature. Through the use of various viruses or hosts and their mutants, viral proteins and host loci important for cell-to-cell and vascular-dependent accumulation have been identified genetically. Expression of viral proteins in transgenic plants for *in trans* complementation studies with mutant viruses has further helped to identify the viral proteins involved in vascular-dependent accumulation. Through the use of antibodies and probes to detect the viral protein and genetic material, respectively, virus accumulation has been visualized in specific tissues and more recently in specific cells over time. The ability to microinject virus or single proteins from the virus and determine their effect on PD between cells has led to a greater understanding of how and where the virus or individual viral proteins manifest their effect on the host to allow virus movement. Lastly, the insertion of marker proteins into virus genomes has made it possible to more easily follow virus movement. The insertion of GFP into viral genomes has made it possible to observe virus trafficking in specific tissues and cells with minimal disruption of the host tissue. However, in spite of these advances we still have not answered some fundamental questions in virus trafficking. The following few paragraphs will highlight what we know and what we still need to find out.

By inoculation or microinjection of viruses, their mutants or individual viral proteins we have identified and, in some cases, mapped the domains or motifs of viral proteins that influence spread. One example is the viral CP. The CP of some viruses is necessary for only vascular-dependent spread while for other viruses it is also necessary for cell-to-cell spread. For other viral proteins, such as the MP and replication-associated proteins, progress has been slower in identifying their role in virus movement due to the confounding effects of their other functions as well as the potential of a virus-induced host defence response when analysing mutants. In spite of this, progress has occurred in defining the roles of some of these proteins in vascular-dependent accumulation. In



many studies the location where the viral protein manifests its effect on vascular-dependent accumulation is unknown. Also, researchers need to determine the abilities of mutant viruses to replicate in young sink tissue. Such studies would differentiate between the virus's ability to enter or exit the vascular tissue and its ability to replicate in sink tissue. Grafting experiments, where transgenic plants expressing a viral protein and nontransformed plants are used as source tissue, will help to define the location where vascular-dependent accumulation is blocked. Finally, it is interesting that we as yet do not know with certainty what form any virus takes while moving through the vascular tissue. Perhaps the use of a battery of mutant viruses and transgenic plants expressing viral proteins in specific cells or the use of a battery of GFP-labelled viruses will provide the answers here.

Researchers have also been successful in genetically defining host factors that affect vascular-dependent accumulation. However, in some instances it is unclear whether the effect is on virus accumulation in general, with a subsequent decrease in virus titre for systemic spread, or on vascular transport directly. For all host resistance loci it will be necessary to determine their effect on the replication of the virus *per se*, and to use grafting experiments and either (1) immunocytochemical or *in situ* hybridization analyses and/or (2) fluorescence analyses of appropriately modified viruses expressing GFP to identify the site of effect (i.e. within the phloem loading trajectory, during SE loading or unloading, or in the phloem unloading trajectory).

Virus movement and photoassimilate movement have been compared for many years and it has been generally accepted, without conclusive evidence, that viruses which move through the phloem enter, move through and exit the phloem similarly to photoassimilates. At least in the phloem unloading trajectory this question has now been addressed through the use of a GFP-expressing virus and dyes to follow photoassimilate transport (Roberts et al. 1997). These researchers determined that virus and photoassimilate come out of the same veins – the third-order or lower order veins of a sink leaf. Although more difficult, it may be possible to follow virus entry into exporting veins by inoculating cells near specific veins (i.e. minor or major veins) with viruses expressing GFP as a marker, isolating these veins by microsurgery and following virus accumulation over time in the inoculated and systemically infected leaves by classical immunocytochemical techniques.

The studies mentioned to this point have dealt exclusively with the movement of virus through phloem tissue of dicotyledonous plants. This is because little is known about virus transport through xylem for any plant species or phloem-dependent accumulation of virus in monocotyledons. There are now multiple examples of viruses which move through xylem and an understanding of this phenomenon may suggest new ways to regulate plant metabolism through transient expression of foreign

proteins from these xylem-associated viruses. The paucity of data on movement of viruses in monocots should be addressed considering the agronomic importance of these plants. An understanding of the mechanism of virus movement in monocots and dicots will allow researchers the ability to devise specific approaches to prevent this movement and thereby supplement currently available resistances in plants.

Finally, the researcher's ability to interpret results from studies on virus spread depend on his/her ability to determine the developmental status and cellular architecture of the host plants at the time of the experiment. As plants are a dynamic entity whose cellular architecture, metabolism and interconnections change over time, the only way to build a foundation of repeatable, meaningful results is to carefully define the status of the plant tissue during the time of the experiment. By combining the use of the new technologies and molecular reagents with the rigorous recording of the physiological status of the host plant, the flow of useful research results on virus trafficking into, through and out of the vascular tissue will continue.

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## **Transgenic Plants in Biochemistry and Plant Physiology**

By Karin Herbers and Uwe Sonnewald

### **1. Introduction**

This chapter is intended to give a general overview of the use of transgenic plants in plant biology. By means of transgenic plants a specific cellular activity can either be increased or reduced by expression of a transgene either in sense or antisense polarity. The objectives associated with these approaches vary; often the function of a gene of interest needs to be elucidated (reversed genetics). Thus, depending on the gene under investigation, aspects in all fields of plant research have been addressed using transgenic plants. For more applied purposes, strategies have been developed to manipulate plant metabolism and metabolic partitioning by the introduction of genes encoding known activities or by the suppression of specific cellular activities with the objective of tailoring biochemical pathways for the production of desired compounds. In addition, transgenic plants have often been created to answer questions in cell biology. For instance, a multitude of plants were transformed with the promoter regions of isolated genes in order to study the temporal and spatial expression of the specific genes. Others were used to investigate the *in vivo* role of putative targeting and signal sequences, the significance of introns, of protein glycosylation, etc. A vast number of transgenic plants, however, have been designed to obtain plants with high resistant properties against pathogens, in particular against viruses. Ectopic expression of genes involved in signal transduction such as homeotic transcriptional factors has also been performed to elucidate their possible role.

It is the authors' objective to give the interested reader an insight into the possibilities of using transgenic plants in plant biochemistry, physiology and cell biology. The overall scope for the use of transgenic plants will broadly be addressed giving representative and/or outstanding examples in all main areas of plant biology, and where possible reviews will be referred to. As the topic of the chapter is very broad, the different fields where transgenic plants are used can only be covered at a superficial level. The first part will deal with currently applied tools to create transgenic plants.

## 2. Plant Transformation

The ability to transfer heterologous genes into plants such that they are stably integrated and transferred to their progeny has revolutionized plant biology. Currently, several approaches are adopted to generate transformed plants; these differ (1) in the choice of DNA delivery, (2) in the choice of the tissue to be transformed, (3) in the selection system for the transgenic tissue and (4) in the regeneration of the transgenic tissue to make a whole plant. For stable transformations either direct gene transfer (Davey et al. 1989) or vector-mediated gene transfer is performed. The most common protocols for dicotyledonous species exploit *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes* vectors as vehicles for foreign genes. Direct gene delivery systems were mainly developed for monocotyledonous plants such as cereals as these are not easily accessible to *Agrobacterium* transformation. A general overview of the actual transformation technology with emphasis on comparing procedures used for different crop plants has recently been given (Christou 1996).

Direct gene transfer can be based on chemical, electrical or mechanical methods. The most commonly used chemical method involves the use of polyethylene glycol (PEG) with the help of which DNA is taken up into plant protoplasts. The mode of action of PEG is largely undefined (Draper et al. 1982; Krens et al. 1982). Electroporation of cells or protoplasts is based on the finding that short electrical pulses reversibly increase the permeability of the plasma membrane to the hydrophilic DNA which under normal circumstances is unable to enter the cell (reviewed in Bates et al. 1988). Using this approach fertile stably transformed plants of oilseed rape (Guerche et al. 1987), rice (Shimamoto et al. 1989) and sugar cane (Arencibia et al. 1995) have been reported. As pectin is implicated as a determinant of cell wall porosity and thus a barrier to DNA uptake (Baron-Epel et al. 1988), cells are often treated with pectinases prior to electroporation. A direct mechanical way to introduce heterologous DNA is to inject it into the nuclei of individual cells or protoplasts using fine glass needles. Using this approach it was possible to transform alfalfa plants (Reich et al. 1986). Another mechanical technique requires silicon-carbide needle-like crystals which penetrate the membranes of cells and may thus allow entry of DNA molecules in their presence. Maize plants have been reported to be successfully transformed by this method (Frame et al. 1994). Yet, the most widespread transformation method for monocots is microprojectile bombardment. Microspheres of gold or tungsten are coated with DNA and accelerated at high velocity, which enables the projectile to penetrate cells and thereby release its DNA. A range of species such as soybean, maize, rice, barley and wheat have been successfully transformed with the help of this method (e.g. Casas et al. 1993). In general, the techniques employing

the *Agrobacterium* system (see below) are preferred to the direct DNA delivery systems discussed, as directly transformed DNA is frequently rearranged or multimerized during insertion into the genome.

The *Agrobacterium* system is the best understood and most widely used vector-based transformation system, particularly for dicot species. It has developed from the understanding that the formation of crown gall tumours by *Agrobacterium* involves the transfer of genetic information from the bacterium to the nucleus of higher plants (summarized in Hooykaas and Schilperoort 1992). This transfer of genetic material from a bacterium to the plant cell is restricted to a definite plasmid-borne DNA segment, the so-called Transfer-DNA (T-DNA) of the bacterium. Crucial progress was made on finding that foreign DNA could be inserted into the T-DNA of the tumour-inducing plasmids (Ti plasmids) and thereby be cotransferred to the plant nucleus (Hernalsteens et al. 1980). This knowledge, together with cognizance of the T-DNA border sequences necessary for the genetic transfer of the T-DNA allowed the construction of "disarmed" Ti plasmids. These possessed the T-DNA border regions, and the oncogenes normally present on the T-DNA were replaced by a genetic marker to monitor the presence of the T-DNA in plants (Zambryski et al. 1983). The disarmed vectors, which were at least 100 kb, were widely used in transferring foreign genes into the plant chromosome until smaller and simpler vectors were constructed to facilitate handling. The "binary" Ti plasmid vectors were developed based on the finding that the T-DNA did not have to be physically linked to the genetic information residing on the remainder of the Ti-plasmid (Hoekema et al. 1983). Thus, the binary vector just retained the T-DNA borders of the original T-DNA while all other functions necessary for the transformation process were present on a Ti-plasmid minus the T-DNA region. This helper plasmid, residing in *Agrobacterium*, replicated and produced the factors necessary for T-DNA transfer. In addition to the T-DNA borders, the binary vector was provided with a useful genetic marker, often the kanamycin resistance gene, and a replicon allowing replication both in *Agrobacterium* and *Escherichia coli*. These basic binary vectors are now widely used in transferring genes into plants (summarized in An 1994). For plant transformation, *Agrobacterium* cells containing the desired construct are incubated with plant-derived explants in sterile tissue culture. Thereafter the explants are regenerated to whole plants in the presence of antibiotics killing the bacteria. More recently, direct delivery systems for *Agrobacterium* have been developed. Stably transformed *Arabidopsis* plants have been obtained after directly injecting *Agrobacterium* cells at served sites at the base of the apical shoots (Chang et al. 1994; Katavic et al. 1994) or by vacuum infiltration of the plants (Bechthold et al. 1993). These in planta transformations avoid time-consuming tissue culture procedures and somaclonal variations often arising during tissue culture.

Dominant selectable marker genes must be part of the integrated DNA to allow selection of transformed plant cells. Most of the genes currently available either confer resistance to antibiotics or herbicides (Walden et al. 1990), such as to the antibiotic kanamycin (Herrera-Estrella et al. 1983) or to the herbicide phosphinothricin (De Block et al. 1987; Rathore et al. 1993). Different markers tend to vary in their effectiveness in different plant species and may also interfere with the plant regeneration process which stimulates continuous search for novel selection markers. For example, recently, Tamura et al. (1995) observed that *Arabidopsis* and tobacco plants expressing the blasticidin S (BS) deaminase from *Aspergillus terreus* were highly resistant against BS.

It can be concluded that there is no universal protocol for the generation of transformed whole plants. For different species the various transformation techniques in combination with different markers have to be empirically investigated in connection with different tissue explants/cells/protoplasts as these differ in their accessibility to the different methods. Also, tissue explants and protoplasts vary in their ability to be regenerated to whole fertile plants. Therefore the transformation of each species is a combinational approach of all methods optimized for the respective species.

### 3. Regulated Expression of Transgenes

The generation of transgenic plants for whatever purpose requires that the gene of interest is expressed in a predictable and desired manner. To this end, promoters of different spatial and temporal specifications are needed. Promoter sequences can be obtained from isolated genes. In this case, specificities of the isolated promoter sequences are investigated in transgenic plants after transfer of chimeric genes consisting of the putative promoter and a reporter gene by means of which the expression can be monitored in plants. Alternatively, promoter trapping experiments in transgenic plants are performed to obtain sequences which drive expression of the introduced reporter gene in desired ways.

#### a) Reporter Genes

The most widespread used reporter gene is  $\beta$ -glucuronidase (GUS) from *Escherichia coli* (Jefferson 1987; Jefferson et al. 1987), but also neomycin phosphotransferase (NPTII), chloramphenicol acetyltransferase (CAT) and luciferase are in frequent use (summarized in Suter-Crazzolara et al. 1995). Others such as phosphinothricin acetyltransferase, catechol oxygenase, gentamycin acetyltransferase, cytosine deaminase and  $\beta$ -galactosidase are less accepted for wide application (for references see

Suter-Crazzolara et al. 1995). The GUS gene not only allows the determination of promoter strength but also the histochemical analysis of the expression in specific cell types or tissues of the transgenic plants. However, there are problems associated with histochemical localizations including diffusion of GUS reaction intermediates (Guivarc'h et al. 1996). Another drawback common also to the other above-mentioned reporter enzymes except for luciferase is that substrates need to penetrate the tissue, which excludes *in vivo* analysis. For these reasons, the green-fluorescent protein (GFP) from jellyfish (*Aequorea victoria*) is receiving increasing attention. As GFP requires only blue or UV light and oxygen and no further exogenous substrates (Chalfie et al. 1994) it is highly desirable as a vital marker during plant growth. Initial studies provided evidence that GFP was valuable as a reporter for virus infections using a potato virus X (PVX) GFP fusion during plant infection experiments (Baulcombe et al. 1995). Due to high viral titers epifluorescence or confocal laser scanning microscopy allowed the detection of the virus in individual infected cells. However, using GFP in stably transformed plants behind the 35S promoter was initially disappointing because of only faint or no fluorescence. Yet, a number of laboratories deal with improving fluorescence emission which might lead to GFP as an excellent marker in the future (Haseloff and Amos 1995, Pang et al. 1996; Reichel et al. 1996).

## b) Promoters

Commonly used promoters in transgenic plants are derived from the T-DNA of *Agrobacterium* and from plant pathogenic viruses. Their significance stems from their ability to result in expression in many different plant tissues, from their strength and broad species compatibility. The promoters driving the expression of the opine biosynthetic genes of the T-DNA from *Agrobacterium* *nos* (nopaline synthase) *ocs* (octopine synthase) or *mas* (mannopine synthase), were isolated and characterized in the early work of plant genetic engineering and are still being used to drive the expression of selection markers in transformed plants.

The most widely used promoter for expression in most plant tissues, and therefore generally referred to as constitutive, is derived from a double-stranded caulimovirus, the cauliflower mosaic virus (CaMV) 35S promoter (Franck et al. 1980). For constitutive expression in monocotyledonous plants actin and ubiquitin promoters have been employed (Zhang et al. 1991; Cornejo et al. 1993; Becker et al. 1994). Artificial promoters have been successfully synthesized to obtain higher expression levels. For instance, the CaMV 35S enhancer has been combined with the wheat  $\alpha$ -amylase promoter or with the maize Adh-1 promoter to generate strong promoters (Last et al. 1991; Omirulleh et al. 1993).

**Table 1.** Promoters of various tissue specificities often employed in manipulating metabolism of dicots

Promoter	Specificity	References
CaMV 35S	"Constitutive"	Benfey et al. (1989), Battraw and Hall (1990)
<i>rolC</i>	Phloem-specific	Schmülling et al. (1989)
<i>ST-LS1</i>	Chloroplast-containing cells	Stockhaus et al. (1989)
Patatin class I (B33)	Tuber-specific	Rocha-Sosa et al. (1989)
ADPG pyrophosphorylase	Stomata	Müller-Röber et al. (1994)
<i>rbcS</i>	Chloroplast-containing cells	Fluhr et al. (1986)
<i>cab</i>	Chloroplast-containing cells	Simpson et al. (1985)

Sometimes it is desired to manipulate plant metabolism only in specific organs, tissues or even cells which requires corresponding promoters. This applies, for example, to studies of sink-to-source interactions in plants which are comprised of processes involved in the biosynthesis of photoassimilates in source leaves, their export and transport to sink organs and the degradation and/or storage in the latter. To facilitate understanding of the role of each partner in this communication system there is a need to manipulate each particular tissue involved differentially. There are principally two approaches to isolate tissue- and developmental-specific promoters. The one usually taken is via isolation of reverse-transcribed mRNAs expressed in the desired way. However, it may also be that apparently constitutive mRNAs may result from the expression of a number of genes with different tissue specificities and that promoters to which deletions have been introduced display altered or restricted tissue specificity (Müller-Röber et al. 1994). The literature on tissue-specific gene expression in plants is extensive; a summary of relevant examples has been published by Benfey and Chua (1989) and Edwards and Coruzzi (1990). Another way to isolate regulating sequences of certain tissue specificity is by promoter- and enhancer-trapping experiments using T-DNA vectors with marker genes which lack promoters or which possess only a minimal CaMV 35S promoter linked to the T-DNA border repeats. Random integration of the transgene behind enhancer and promoter sequences will allow expression of the marker, in which case a regulating sequence can be identified and subsequently isolated (André et al. 1986; Koncz et al. 1989, 1990; Fobert et al. 1994 and references therein). Promoters of different tissue specificities most often used for generating transgenic dicotyledonous plants are listed in Table 1.

Unfortunately, promoter sequences of defined tissue specificity are often also subject to additional developmental and environmental con-

trol. For instance, the tuber-specific patatin promoter B33 has been found to be inducible in leaves by sucrose (Rocha-Sosa et al. 1989), or, the genes encoding the small subunit of ribulose 1,5-bisphosphate carboxylase oxygenase (RUBISCO, *rbcS*), the chlorophyll a/b binding proteins (*cab*) and a component of the oxygen-evolving complex of photosystem II (ST-LS1) are strongly regulated by light (Edwards and Coruzzi 1990, and references therein). To rule out that ectopic expression of a transgene is also governed by these additional parameters, inducible promoters are needed. Inducible promoters, moreover, would allow the expression of possibly harmful transgenes, and direct effects caused by the transgene can be studied immediately after induction of expression, avoiding pleiotropy due to plant adaptations to the transgene. Endogenous plant promoters responding to external stimuli such as heat, wounding, nitrate, sugars, plant hormones or cold have been characterized and could be used to control ectopic expression of transgenes. However, a disadvantage is that these stimuli affect transcription of a number of endogenous plant genes as well. An alternative is to place the regulatory system under controlling stimuli usually not encountered in plants (for review see Ward et al. 1993).

Two main concepts for inducible expression systems have been developed. These are based either on a promoter-repressing or on a promoter-activating system. In the latter, a minimal promoter allowing correct transcription initiation is fused to an activator-recognition site directing the expression of a target sequence. One example is the steroid-inducible system from mammals. The minimal promoter is fused to the glucocorticoid response element (GRE). In the presence of a glucocorticoid (dexamethasone) the glucocorticoid receptor expressed from a constitutive promoter binds to the GRE and activates transcription (Schena et al. 1991). Similarly, in the copper-induced expression system from yeast the ACE1 regulatory protein is activated by copper and binds to the metallothionein promoter (Mett et al. 1993). GUS activity of transgenic tobacco was increased 50-fold upon treatment of the plants with copper (Mett et al. 1993). The promoter-repressing systems are borrowed from bacterial regulatory operons. The gene encoding the target protein is placed under the control of a promoter of desired strength and specificity to which one or more operator sequences have been added. The corresponding repressor is expressed in the same cell types resulting in binding of the repressor to its operator, thereby suppressing transcription. Derepression is obtained by treatment of the tissue with an inducer. Using the Lac repressor-operator system, isopropylthiogalactoside (IPTG)-inducible transcription was obtained in protoplasts from transgenic plants (Wilde et al. 1992). Gatz et al. (1992) succeeded in establishing a tightly repressible system by combining the CaMV 35S promoter with three operator sequences with DNA-binding activity to the *Tn10*-encoded tetracyclin repressor (TetR). The TetR is



expressed from the CaMV 35S promoter in the same cells. Using low amounts of tetracycline (1 mg/l) leads to a 200- to 500-fold induction of promoter activity in intact tobacco plants (Gatz et al. 1992).

In the field of promoter studies questions of basic research and use of promoters to drive heterologous genes are tightly entwined because the specificities of promoters, unravelled in transgenic plants, are prerequisites for fine-tuned and controllable impacts of heterologous genes in transgenic plants. Further studies of promoters in transgenic plants allowed to ascribe of specific functions to enzyme isoforms due to their differential expression. Deletion analyses permitted the identification of regulatory sequences of promoters by means of which it became possible to isolate transcription factors binding to them. The enormous significance of transgenic plants for promoter studies is reflected by the fact that most transgenics created up until today were transformed with promoter-reporter gene constructs.

### c) Correct Subcellular Targeting

As eukaryotic cells carry out metabolic processes in diverse subcellular compartments it is often necessary to direct heterologous proteins into a specific organelle to make use of compartmentalized substrates. For instance, diverting flux from acetyl-CoA to polyhydroxybutyrate (PHB) in plastids resulted in considerable PHB production (14% of dry weight of senescing leaves) whereas in the cytosol hardly any PHB synthesis took place (Somerville 1996).

With the exception of a few plastidic and mitochondrial proteins, the polypeptides present in organelles are encoded by nuclear DNA, and translation of the respective mRNAs occurs on free cytosolic or on endoplasmic reticulum (ER)-bound ribosomes. Transport of the proteins to their destination depends on the presence of inherent targeting signals (Fig. 1). Heterologous proteins must therefore be expressed as fusion proteins containing a suitable targeting sequence to be properly delivered to the organelle where they are intended to act.

Proteins of the ER, the Golgi apparatus, the vacuole, the plasma membrane and extracellular space are synthesized on ER-bound ribosomes as pre-proteins with N-terminal hydrophobic signal peptides allowing entry of the nascent polypeptide into the lumen of the ER (for reviews see Chrispeels 1991; Gomord and Faye 1996). In order to be retained in the ER, proteins require a specific retention signal which has been identified as HDEL and KDEL C-terminal extensions, otherwise they are transported to the Golgi apparatus via vesicles (Gomord and Faye 1996). For the Golgi resident membrane proteins it has been found that length but not any specific sequence of their transmembrane

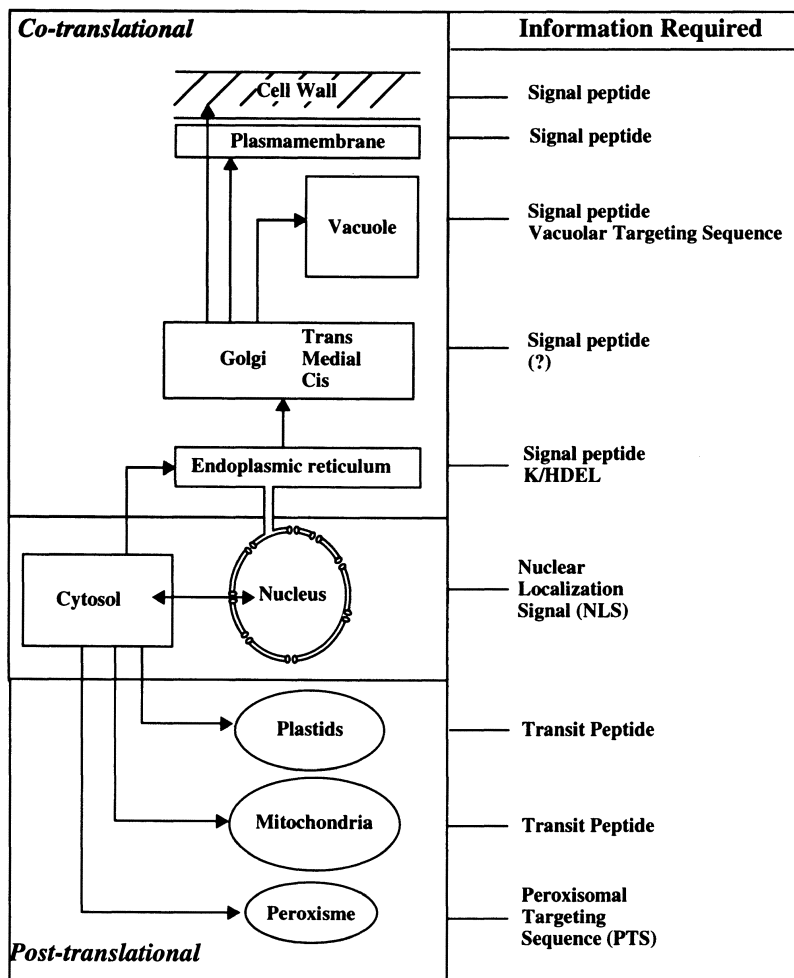


Fig. 1. Intracellular protein targeting in plants

domain plays a crucial role in retention (Gomord and Faye 1996). Unless carrying a specific vacuolar targeting signal which will destine them for the vacuole, proteins are transported to the extracellular compartment from the trans-Golgi network (for reviews see Nakamura and Matsuoka 1993; Neuhaus 1996). Three different types of vacuolar targeting peptides have been identified. These are either N- or C-terminal propeptides or are located within the sequence of the mature protein (Neuhaus 1996, and references therein). Nuclear-encoded mitochondrial and plastidic

polypeptides are synthesized as precursor proteins in the cytosol containing N-terminal transit peptides (for reviews see Smeekens et al. 1990; Glick et al. 1992). Representative examples of mitochondrial and chloroplast targeting peptides have been compiled by von Heijne et al. (1989).

The tools to study targeting of proteins in plant cells have mainly been (1) *in vitro* uptake of the respective proteins in isolated organelles and (2) fractionation studies of protoplasts transiently expressing the protein under investigation. The expression of chimeric genes consisting of putatively identified targeting sequences as fusions with easily monitored markers in transgenic plants greatly expanded knowledge in this area of cell biology. Nowadays, targeting signals thus identified are routinely exploited to direct proteins to the desired subcellular compartments.

#### **4. Ectopic Expression of Transgenes: Approaches**

There are two principal ways to create transgenic plants for studying aspects in plant physiology. Either the expression level of endogenous plant genes is altered, or heterologous genes (either plant-derived or from unrelated organisms) are ectopically expressed. The different strategies and objectives that may be associated with these manipulations have recently been summarized (Herbers and Sonnewald 1996). Down-regulation of endogenous genes can either be achieved by antisense inhibition or by co-suppression. Antisense inhibition occurs via expressing the RNA strand complementary to the sequence of the target mRNA which had first been reported by van der Krol et al. (1988). Since then this concept has widely been used although the mechanism is not well understood (for review see Mol et al. 1990). Down-regulation by co-suppression can be obtained by expression of homologous sense transcripts also termed in a mechanistic context as homology-dependent gene silencing (Napoli et al. 1990). It appears that there are two classes of homology-dependent gene silencing. The first probably involves cytoplasmic RNA turnover and the second is associated with transcriptional inactivation, often accompanied by increased cytosine methylation (for reviews see Matzke et al. 1996, Meyer and Saedler 1996). Altering levels of endogenous genes via the antisense or co-suppression approach provides useful alternatives to the use of mutants generated by mutagenic agents such as chemicals (ethyl methane sulphonate, EMS; N-methyl-N'-nitro-N-nitrosoguanidine, MNNG, sodium azide), UV and gamma radiation as well as by tagging approaches using T-DNA or transposons:

1. Many species are not easily mutagenized due to large genomes and complicated genetics.

2. Considerable biochemical variation between plant species may occur in certain areas of metabolism (such as lipid composition of membranes, sink-to-source interactions) which does not allow easy transfer of knowledge from *Arabidopsis* which is amenable to these approaches to, for instance, a potato plant.
3. Transgenic plants generated by reverse genetics represent a set of mutants with gradually decreased or increased activities whereas induced mutations usually lead to loss-of-function mutants.
4. It is nearly impossible to detect mutations in multi-copy genes while the antisense or co-suppression approach may affect all genes of multigene families provided the degree of homology is sufficient (Rodermeil et al. 1988).
5. By means of molecular genetics transgenic plants can be designed such that target enzymes can be inhibited in a tissue- or cell-specific manner with the help of adequate promoters (Leidreiter et al. 1995), and manipulations (in case of overexpression) can even be directed to different cellular compartments (e.g. Nawrath et al. 1995).
6. In case of potential lethality an inducible promoter can be used for expression while mutants generated by mutagenic agents would not survive.
7. Detection of induced mutants requires a screening system feasible for a large number of plants which may not be available.
8. Metabolite concentrations or pathways can be altered by expression of genes encoding proteins not encountered in plants, or by expression of genes encoding enzymes with no or altered regulatory properties. Even novel pathways may be introduced into transgenic plants by these means (reviewed in Herbers and Sonnewald 1996).

Despite this multitude of advantages, precautions need to be taken when working on transgenic plants. Due to random integration events of the transgene, insertional mutants with altered phenotypes not based on the transgene may occur. In addition, during plant tissue culture genomic reorganizations sometimes take place ("somaclonal variations") leading to altered phenotypes also independent from the introduced transgene. For these reasons several independent transgenic lines need to be analysed in order to verify effects observed on the transgenic plants.

## 5. Use of Transgenic Plants in Plant Biology: A Broad Overview

### a) Cell Biology

As mentioned in Sections 3.b and 3.c a number of questions in cell biology have been and are addressed with the help of transgenic plants: gene regulation and intracellular transport processes. For these studies either regulating promoter/enhancer sequences and target/signal sequences

within adequately constructed chimeric gene constructs are introduced into transgenic plants. Also, other cell biological questions have been addressed with the help of transgenic plants. These include studies of gene expression not only at the level of transcription but also at post-transcriptional steps such as splicing, 3'end processing, polyadenylation of pre-mRNAs in the nucleus, nucleocytoplasmic transport, translation and mRNA stability. For all these studies different parts of the genes under study, such as 5' and 3'untranslated sequences, introns, mutated translational initiation sites and polyadenylation signals, have been analysed *in vivo*. In addition, the putative role of glycosylation of proteins which enter the secretory pathway has also been investigated in transgenic plants. By means of site-directed mutagenesis glycosylation sites had been removed from the protein under study and targeting, stability and function of the respective protein were subsequently analysed in transgenic plants. Thus, for instance, it was found that the removal of glycosylation sites from the seed-specific phythemagglutinin and tuber-specific patatin proteins had no influence on stability, intracellular transport and other characteristics of the respective proteins in transgenic tobacco plants (Voelker et al. 1989; Sonnewald et al. 1990).

#### b) Primary and Secondary Metabolism

Using the antisense technology or the co-suppression approach, transgenic plants have been created to study the role of individual enzymes in primary and secondary metabolism and in partitioning of metabolites between primary and secondary metabolism (Herbers and Sonnewald 1996). Carbohydrate metabolism has been extensively manipulated, in particular with regard to understanding determinants of sink-and-source strengths which have been subject to a number of recent reviews (Sonnewald and Willmitzer 1992; Sonnewald et al. 1994; Frommer and Sonnewald 1995, Müller-Röber and Koßmann 1995; Stitt and Sonnewald 1995). The increasing number of target genes in other areas of primary and secondary metabolism has led to a steady increase in reports on corresponding transgenic plants. Studies on function and regulation of lipid composition by means of transgenic plants in comparison with classical mutants have recently been reviewed (Gibson et al. 1994). In this field of metabolism, transgenic plants are of particular significance due to high lipid variations between different plant species (Gibson et al. 1994). The manipulation of fatty acid composition with respect to chain length and degree of saturation, particularly in seeds, is of considerable commercial interest because of the varying processing characteristics of the resultant oils and fats (reviewed by Murphy 1996; Chrispeels and Sonnewald 1997). Furthermore, plants differing in their fatty acid composition are also of interest to study chilling and cold tolerance.

The nitrate assimilatory pathways in plants has been a matter of intense research for the last 40 years because of its fundamental role in plant nutrition (reviewed in Hoff et al. 1994). Nitrate is actively taken up into roots via nitrate transporter(s) where it is either stored in the vacuole, reduced in the cytosol or transported to the leaves. Reduction to ammonia occurs in two steps with NADH or NADPH as electron donors. The reduction from nitrate to nitrite is catalyzed by nitrate reductase (NR), the conversion from nitrite to ammonia by nitrite reductase (NiR). Ammonia is then incorporated into amino acids by glutamine synthetase (GS) and glutamate synthase (GOGAT). The isolation of cDNA and genomic clones encoding NR and NiR and of a nitrate transporter enabled molecular studies on the regulation of these genes (Hoff et al. 1994 and references therein). Classical mutants and transgenic plants revealed the complex regulation of nitrate assimilation which is stimulated by nitrate, soluble sugars and cytokinins, repressed by a high glutamine-to-glutamate ratio and, furthermore, subject to a circadian rhythm (Hoff et al. 1994). As the transgenic plants offer the possibility that the activity of an enzyme can be gradually decreased, the impact of a given enzyme on flux through the respective pathway can be estimated. Interestingly, for NR activity it has been shown that contrary to expectations transgenic tobacco plants expressing as low as 10% of wildtype activity grew as well as wild-type plants under greenhouse conditions (Dorbe et al. 1992).

Subsequent steps leading to the synthesis of amino acids have also been subject to thorough genetic and molecular analysis. This interest is closely connected to the realization that important classes of herbicides (phosphinothricins, glyphosates, imidazolinones, sulphonylureas and triazolopyrimidines) act by inhibition of amino acid biosynthesis. Different concepts to create herbicide-resistant plants (also used as selection markers during plant transformation, see Sect. 2) have been followed by means of transgenic plants: (1) Increasing the activity of the target enzyme; (2) introducing modified target or heterologous enzymes which are not inhibited by the herbicide; (3) introducing activities that modify the herbicide, thereby leading to its inactivation (Piruzian et al. 1988). For example, overproduction of alfalfa GS in transgenic tobacco plants amounted to a 5-fold increase in specific GS activity leading to a 20-fold increase in resistance to the GS inhibitor L-phosphinothricin ("Basta") in in vitro culture (Eckes et al. 1989). However, later reports of overproducing GS in transgenic plants have been less positive (summarized in Lea and Forde 1994). The alternative way of detoxification has been pursued by expressing the *bar* gene from *Streptomyces hygroscopicus* encoding a phosphinothricin acetyltransferase (De Block et al. 1987). Acetohydroxy acid synthase (AHAS) catalyses the first step in the biosynthesis of the branched-chain amino acids valine, isoleucine and leucine. This enzyme is inhibited by sulphonylureas, imidazolinones

and triazolopyrimidines. A mutant allele of AHAS responsible for sulphonylurea resistance in a *Brassica napus* cell line was transferred into transgenic tobacco plants where it conferred strong resistance to the three classes of herbicides (Hattori et al. 1995). The amino acids tryptophan, tyrosine and phenylalanine are synthesized via the shikimate pathway. The herbicide glyphosate ("Roundup") is an inhibitor of the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) and thus prevents the conversion of shikimate to chorismate. The endeavours to engineer crop plants with tolerance to glyphosate have been reviewed by Barry et al. (1992).

A range of secondary compounds including lignin, flavonoids, phytoalexins and alkaloids is derived from the aromatic amino acids. Therefore, the shikimate pathway is considered to be an important link between primary and secondary metabolism. It has been estimated that up to 60% of the dry weight of a plant is derived from carbon flux through the shikimate pathway (Jensen 1986). Lignin represents the most abundant of these compounds and the second most abundant organic compound on earth. This renders it highly attractive for genetically modifying its structure in order to (1) increase its digestibility in animal feed and (2) create wood with specified characteristics for manufacturing. Furthermore, increasing flux into lignin compounds might help to contribute to plants' preformed resistance against pathogens, and, on the other hand, diverting flux from lignin into competing pathways could lead to plants usable as efficient production systems for corresponding compounds. First attempt to alter lignin composition by repressing biosynthetic enzymes have been reported (Dwivedi et al. 1994; Halpin et al. 1994; Atanassova et al. 1995; McIntyre et al. 1996). Co-suppression of phenylalanine ammonia-lyase (PAL) activity in tobacco plants ranging from wildtype levels to levels of 0.2% of wildtype revealed that PAL was the dominant step regulating overall flux into phenylpropanoid compounds in leaves (Bate et al. 1994). By means of these plants Maher et al. (1994) were able to show that performed phenylpropanoid products are essential for maintaining disease limitation against the fungal pathogen *Cercospora nicotianae*.

The committed step in flavonoid biosynthesis is catalyzed by chalcone synthase (CHS). Transfer of a chimeric gene containing the CaMV 35S promoter driving the antisense CHS gene from *Petunia hybrida* in transgenic tobacco and *Petunia* plants resulted in inhibition of flower pigmentation (van der Krol et al. 1988). Repression or induction of single biosynthetic enzymes has not been the only approach to alter flower coloration, which is of commercial interest in the cut-flower industry. As a number of regulatory genes involved in flower pigmentation have been isolated it has been possible to express anthocyanin pathway-specific transcriptional activators in transgenic plants. Thus, transfer of R and

C1 genes from maize into *Arabidopsis* and tobacco plants activated anthocyanin production in the latter (Lloyd et al. 1992).

Other biosynthetic pathways such as carotenoid and chlorophyll biosynthesis (e.g. Misawa et al. 1993; Kruse et al. 1995) have also been the subjects of genetic manipulations but will not be considered here.

### c) Pattern Formation, Growth and Development

Transgenic plants have been valuable to study plant growth, development and pattern formation. These topics have been addressed mainly by two different strategies: (1) influencing levels of phytohormones/signal compounds and (2) overexpression of regulatory genes. Hormonal effects on plants have been analysed by either suppressing or increasing endogenous levels of hormones. This has been achieved by altered expression of synthesizing or degrading activities, or via overexpression of enzymes involved in converting the hormones into inactive compounds (Table 2). Recently, a novel strategy has been developed. Single-chain Fv antibodies have been expressed in transgenic tobacco and potato plants to reduce the amount of functional unbound abscisic acid (ABA, Artsaenko et al. 1995). Traditionally, research on phytohormones was conducted by the use of hormone-deficient or hormone-insensitive mutants and by the exogenous application of phytohormones to plants or plant tissues. Both approaches have provided a wealth of information but they suffer from limitations. (1) For some phytohormones, such as auxin, cytokinin and ethylene, no hormone-deficient mutants exist. This might be due to the presence of several genes for most or all steps in the biosynthesis of these hormones or mutants with low levels of auxin or cytokinin might be inviable. To the authors' knowledge there are also no mutants available for jasmonic acid, salicylic acid and systemin. (2) External application of signalling compounds requires uptake and transport, the degree of which is often unknown.

Most work on phytohormones involving transgenics has been carried out on altering levels of auxin and cytokinins. This work emanated from findings that *A. tumefaciens* and *A. rhizogenes* are the causative agents of crown gall and hairy root disease, respectively. The tumorous growths associated with these diseases are a consequence of altered hormonal metabolism in plant cells transformed with the T-DNA of these bacteria. The T-DNA of *A. tumefaciens* contains genes encoding enzymes which are involved in the biosynthesis of auxin (*iaaM* and *iaaH*) and cytokinin (*ipt*) whereas those of *A. rhizogenes* encode glucosidase activities that may liberate cytokinin (*rolC*) and auxin (*rolB*) from their inactive glucoside conjugates (Estruch et al. 1991a,b). The function of the *rolA* gene which also contributes to morphological changes in the development of



Table 2. Transgenes affecting hormone physiology

Hormone	Transgene	Biochemical effects	Enzymatic activity	References
ABA	Anti-ABA antibodies	Inhibition of action	Binding of single chain antibodies to ABA	Artsaenko et al. (1995)
Auxin	<i>Pseudomonas syringae</i> <i>iaaL</i>	Inactivation	Conjugates IAA to Lys	Romano et al. (1991), Spena et al. (1991)
	<i>Arabidopsis</i> nitrilase II	Increased biosynthesis	Converts indole-3-acetonitrile to IAA	Schmidt et al. (1996)
	<i>A. tumefaciens</i> <i>iaaM</i>	Increased biosynthesis	Converts Trp to IAA	Klee et al. (1987)
	<i>A. tumefaciens</i> <i>iaaM</i> + <i>iaaH</i> <i>A. rhizogenes</i> <i>rolB</i>	Increased biosynthesis Increased sensitivity to auxin	Converts Trp to IAA Hydrolyses indoxyl glucosides (in vitro)	Sitbon et al. (1991) Schmülling et al. (1988)
Cytokinin	<i>A. tumefaciens</i> <i>ipt</i> <i>A. rhizogenes</i> <i>rolC</i>	Inactivation Overall change in phytohormones	Condensates IP-PP to AMP Hydrolyses cytokinin glucosides (in vitro)	Smigocki and Owens (1988) Schmülling et al. (1988)
Ethylene	<i>Pseudomonas</i> ACC deaminase	Inhibition of biosynthesis by diverting flux	Converts ACC to a-ketobutyric acid	Klee et al. (1991)
	Tomato ACC oxidase	Antisense inhibition of biosynthesis	Blocks conversion of ACC to ethylene	Hamilton et al. (1990)
	Tomato ACC synthase	Antisense inhibition of biosynthesis	Blocks conversion from SAM to ACC	Oeller et al. (1991)
	T3 SAM hydrolase	Inhibition of biosynthesis by diverting flux	Converts SAM to MTA and homoserine	Good et al. (1994)

Table 2 (continued)

Hormone	Transgene	Biochemical effects	Enzymatic activity	References
Jasmonic acid	Flax aos	Increased biosynthesis	Converts hydroperoxide to allene epoxide	Harms et al. (1995)
	<i>Arabidopsis</i> LOX II	Co-suppression	Reduced formation of jasmonic acid	Bell et al. (1995)
Salicylic acid	<i>Pseudomonas putida nahG</i>	Inactivation	Hydroxylates salicylic acid to catechol	Gaffney et al. (1993)
Systemin	Tomato prosystemin	Antisense inhibition	Reduced formation of systemin	McGurl et al. (1992)
	Tomato prosystemin	Overexpression	Increased formation of systemin	McGurl et al. (1994)

ABA, abscisic acid; ACC, 1-aminocyclopropane-1-carboxylic acid; AMP, adenosine monophosphate; aos, allene oxide synthase; IAA, indole-3-acetic acid; *iaaH*, indoleacetamide hydrolase; *iaaL*, indoleacetic acid-lysine synthetase; *iaaM*, tryptophan monooxygenase; IAM, indoleacetamide; IP-PP, isopentenyl pyrophosphate; *ipt*, isopentenyltransferase; LOX, lipoxygenase; Lys, lysine; MTA, methylthioadenosine; SAM, S-adenosyl methionine; Trp, tryptophan.

root-hair disease is less defined. These T-DNA residing genes have been expressed in transgenic plants either individually or in different combinations, using either their own, the much stronger CaMV 35S and 19S promoters or an inducible heat-shock promoter. The different genetic approaches to influence cytokinin and auxin levels and their biochemical/morphological effects on the plants have been summarized in comprehensive reviews (Brzobohaty et al. 1994; Hobbie and Estelle 1994, Hobbie et al. 1994). Many of the created transgenic plants confirmed what had been known from the classical type of experiments. Yet, a surprising outcome was that plants expressing heterologous biosynthetic activities or hormone glucoside hydrolysing enzymes were highly flexible and adjusted levels of functional auxin and cytokinins to a considerable degree.

Ethylene, on the other hand, could be reduced to 5 and even 0.5% of wildtype levels in tomato fruits by successfully suppressing the biosynthetic enzymes 1-aminocyclopropane-1-carboxylic acid (ACC) oxidase and ACC synthase, respectively, using the antisense approach (Hamilton et al. 1990; Oeller et al. 1991; see also Table 2). These transgenic plants have been thoroughly characterized with emphasis on fruit ripening, fruit texture and senescence and the reader is referred to recent informative reviews on this topic (Gray et al. 1992, 1994). Most instructive so far have been investigations of the signalling compounds salicylic acid (SA), systemin and jasmonic acid (JA). These compounds do not significantly contribute to growth and development of plants but appear to have pivotal roles when plants suffer from biotic or abiotic stresses. For example, SA which is synthesized from cinnamic acid was found to increase manifold in tobacco, cucumber and *Arabidopsis* plants after pathogen infection (see Ryals et al. 1996 for references). Furthermore, its increase correlated with systemic acquired resistance (SAR), a resistant state of plants acquired after pathogen attack, which protects plants against further infections by the same or even unrelated pathogens (Ryals et al. 1996). Gaffney et al. (1993) created transgenic plants which expressed a bacterial gene (*nahG*) encoding salicylate hydroxylase which catalyzes the conversion from SA to catechol (Table 2). These plants were unable to accumulate SA upon pathogen attack and they were, moreover, incapable of developing an SAR response, thus providing compelling evidence that SA mediated SAR (Gaffney et al. 1993). A number of *Arabidopsis* mutants that are either constitutively activated for SAR or unable to mount the SAR in response to pathogens have been isolated (Ryals et al. 1996). The *nahG* expressing plants have been most valuable in positioning the *Arabidopsis* mutants along the signal transduction pathway in SAR and defining their dependence on SA (Ryals et al. 1996). Similarly, the role of JA and its methyl ester (MJ) in the induction of wound-induced genes has been investigated using transgenic plants. The external application of JA or MJ to plants revealed that a

number of genes that are wound-inducible, such as the proteinase inhibitor genes *pinI* and *pinII* in potato and tomato plants as well as vegetative storage proteins (*vsp*) in soybean and *Arabidopsis* plants, are inducible by JA and MJ (Farmer and Ryan 1990; Mason and Mullet 1990, Staswick 1990). Mechanical wounding itself leads to increased levels of JA in soybean hypocotyls (Creelman et al. 1992). JA and MJ are derived from fatty acids by lipoxygenases-dependent oxidation (Vick and Zimmermann 1984). To analyse whether the wound response is mediated by JA, transgenic *Arabidopsis* plants were generated where co-suppression reduced lipoxygenase II (LOXII) activity (Bell et al. 1995). These plants were unable to synthesize JA and, furthermore, to accumulate *vsp* mRNA in response to wounding (Bell et al. 1995), suggesting that wound-induced JA is involved in the regulation of the *vsp* gene. These examples show the significance of plants generated by reversed genetics to create well-defined mutants and to elucidate hormonal involvement in specified signal transduction pathways.

In plant development there are two main areas in which transgenic plants have been made use of: (1) photomorphogenesis (particularly with regard to seedling development) and (2) cell fate and morphogenesis independent of light, in particular with regard to flower development. Light plays a crucial role in all processes associated with plant growth and development. One important challenge to the scientific community is to understand the light-regulated signal transduction pathways. Photomorphogenesis has been intensively studied using photomorphogenic mutants falling into different groups: (1) the photoreceptor mutants (affected in photoperception by phytochromes, by blue-light/UV-A and UV-B and (2) non-photoreceptor mutants with reduced photosensitivity (*hy5*, *fhy1*, *fhy3*, *blu1*, *blu2*, *blu3*), or with normal seedling development despite darkness (*cop*, *det*, *fus*) (reviewed in Quail 1994). Several of these genes have been cloned (see Quail 1994 for references) and studied in transgenic plants to investigate the putative function allocated to the respective gene from the classical mutants (e.g. McNellis et al. 1994; Whitelam and Harberd 1994 for phytochromes).

Cell morphogenesis comprises two processes, the first is that an undifferentiated cell recognizes its fate dependent on its position and, second, that it differentiates according to its adopted fate. Cell fate mutants with altered leaf development in maize (*knotted1*, *kn1*; *rough sheath1*; *rs1*; *liguleless*, *lg3*, *lg4*) and epidermal fate mutants (*glabra1*; *gl1*; *transparent testa glabra*, *ttg*; *triptychon*, *try*) in *Arabidopsis* have long been identified (reviewed in Schiefelbein 1994). Also, in flowers of *Arabidopsis* and *Antirrhinum majus*, a number of cell fate mutants have been characterized that cause cells to misinterpret their position in their organ primordium, resulting in differentiation into inappropriate cell types and thus organ types (for references see Mandel et al. 1992). From these diverse so-called homeotic mutants a genetic model for the developing

flower has been evolved which has been checked for validity by ectopic expression of the homeotic genes (for references see Ramachandran et al. 1994). The homeotic genes of flower development belong to the MADS-genes transcriptional factors whose proteins contain a region (the MADS box) with sequence homology to MCM1 (from *Saccharomyces cerevisiae*), AGAMOUS (from *A. thaliana*); DEFICIENS (from *A. majus*) and SRF (from *Homo sapiens*). The *kn1* gene encodes another class of transcriptional factors containing a homeobox domain also conserved between animals and plants. The phenotype of transgenic tobacco plants expressing maize *kn1* gave additional evidence that this gene is involved in determining leaf cell fate (Sinha et al. 1993). Due to sequence homology many different homeobox-containing genes have been isolated from different plant species since then, assuming that they would possess a regulating homeotic function in plant development. Ectopic expression of these unknown genes has largely confirmed this hypothesis of their role as master switches in developmental processes (Schena et al. 1993; Lincoln et al. 1994; Aoyama et al. 1995; Sato et al. 1996). Other famous examples of understanding the function of regulatory genes concern the flower-meristem identity genes. Flower-meristem identity genes have been recognized in *Arabidopsis* by mutants (CAULIFLOWER, *cal*; APETALA1, *ap1*; LEAFY, *lfy*) which lead to the development of shoots or shoot-like structures from meristems that would normally form flowers. To further assess the individual role of each of these genes, *lfy* has been constitutively expressed in *Arabidopsis* and aspen plants (Weigel and Nilsson 1995) and *ap1* in *Arabidopsis* plants (Mandel and Yanofsky 1995). In both cases, flower development was induced precociously and both proteins were able to transform apical and lateral shoots into flowers. Thus, both *ap1* and *lfy* proteins were independently sufficient to convert shoot meristems into floral meristems and this capacity was conserved between *Arabidopsis* and aspen species (Mandel and Yanofsky 1995, Weigel and Nilsson 1995).

## 6. Abiotic and Biotic Stresses: Resistance

Stress physiology is one of the main areas of genetic engineering research. As plants are immobile they have developed myriad mechanisms to protect themselves from or adapt to all sorts of abiotic and biotic stress forms. Because of the multitude of different stresses and plant protection mechanisms expressed via transgenic plants, it is not possible to discuss any of them in detail.

### a) Abiotic Stresses

Different environmental adverse conditions such as drought, salinity and cold temperatures may lead to cellular dehydration and can thus be considered as different forms of osmotic stress. Other abiotic stresses encountered by plants are exposure to heat, elevated levels of ozone, to UV light, anaerobiosis and heavy metals. Most molecular genetic strategies of identifying components which might assist a plant to resist these adverse conditions can be summarized as follows: (1) the biochemical and molecular state of a plant under normal circumstances is compared with a plant under hostile conditions or, alternatively, cultivars or species that are tolerant to certain adverse conditions are compared at the biochemical and molecular level with plants that are sensitive. Another possibility to induce a differential state may consist in pretreatment of plants with compounds known to mediate a certain degree of resistance against the external factor. For instance, it has been shown that desiccation tolerance of *Craterostigma plantagineum* can be obtained by treating the callus with exogenous abscisic acid (ABA) prior to desiccation (Bartels et al. 1990), or, exogenous application of tobacco plants with SA can induce SAR (White 1979). (2) Differentially expressed mRNAs identified in this way are isolated from the tolerant plant or from the plant exposed to stress conditions. (3) The characterized genes are then reintroduced into the sensitive plant to evaluate whether the gene under investigation contributes to increased tolerance to the hostile condition. Alternatively, genes that might lead to the biochemical state (such as membrane lipid composition, degree of fatty acid saturation, different levels of osmoprotectants, etc.) contributing to a certain degree of tolerance have been isolated either from the more tolerant plant species or any other organism and investigated in the same way as described in this section under (3). By applying these molecular biological techniques or variations thereof, many genes involved in improving resistance to physical stresses have been cloned (Sachs and Ho 1986) and analysed in transgenic plants (reviewed in Bartels and Nelson 1994; Chrispeels and Sonnewald 1997).

### b) Biotic Stresses

Infections by fungi, bacteria, viruses, insects and nematodes exert substantial biotic stress on plants. Plants have developed sophisticated mechanisms that render them resistant against most pathogens. For most of the individual traits attributing to resistance no mutants are available such that many genes were transferred and characterized in transgenic plants. There is a considerable number of genes that helped to engineer elevated resistance against a particular pathogen. The most

Table 3. Important examples of transgenes affecting pathogens

Hormone	Transgene	Biochemical effects	References
Insect			
	<i>B.t. cry</i>	Midgut receptor binding	van der Salm et al. (1994 and references therein)
	Cowpea trypsin inhibitor <i>pin</i> I and <i>pin</i> II	Inhibition of trypsin Inhibition of chymotrypsin and trypsin	Hilder et al. (1987) Johnson et al. (1989)
Virus			
	Viral genes	PD resistance mechanisms	Baulcombe (1996), Fitch and Beachy (1993)
	2-5Aase and RNaseL	Mammalian 2',5'A antiviral system	Ogawa et al. (1996), Mitra et al. (1996)
	Pokeweed RIP	Inhibition of translation and/or preventing virus from entering the cell	Lodge et al. (1993)
	Yeast <i>pac1</i>	Hydrolysis of double-stranded RNA	Watanabe et al. (1995)
	Single-chain Fv antibody	High affinity for AMCV coat protein	Tavladoraki et al. (1993)
	Tobacco N	Confers resistance against TMV	Whitham et al. (1996)
Fungus/Bacteria			
	Radish AFP2	Cysteine-rich "defensin"	Terras et al. (1995)
	Barley RIP	Inhibition of translation	Logemann et al. (1992)
	PR-proteins	Cell wall hydrolysing activities and others unknown	Jach et al. (1995), Zhu et al. (1996 and references therein)
	Grapevine stilbene synthase	Biosynthesis of phytoalexin resveratrol	Hain et al. (1993)
	Tomato <i>pto</i>	Resistance against <i>Pst</i> harbouring avrPto	Rommens et al. (1995), Thilmony et al. (1995)

AFP, antifungal protein; AMCV, artichoke mottled crinkled virus; 2-5Aase, 2',5'oligoadenylate synthetase; *B.t. cry*, *Bacillus thuringiensis* crystal protein encoding genes; PD, pathogen-derived; *pin* I and II, proteinase inhibitors I and II; PR, pathogenesis-related; *Pst*, *Pseudomonas syringae* pv. *tomato*; RIP, ribosome-inactivating protein; RNaseL, ribonuclease L; TMV, tobacco mosaic virus.

commonly applied ways of engineering and studying resistance responses against diverse pathogens are addressed below (see also Table 3).

#### $\alpha$ ) Insect Tolerance

*Bacillus thuringiensis* (*B.t.*) produces highly insecticidal proteinaceous crystals which display different insecticidal spectra. A number of different *cry* genes encoding crystal proteins have been isolated and characterized. The insecticidal proteins bind to different receptors present on the membrane of midgut epithelial cells of insects. Insect-resistant transgenic plants of several different species have been obtained by expressing *B.t.* *cry* genes. As expression of the bacterial genes in planta was rather low, reaching levels of about 0.001% of total leaf protein, much effort has been devoted to modifying the genes to achieve higher expression levels. Improved expression in plants was achieved by extensive modification of the coding region without altering the amino acid sequence (Perlak et al. 1993; van der Salm et al. 1994, and references therein). Alternatively, fusions, such as translational fusions between different *cry* genes, were performed to achieve greater transcript stability, broader resistance and an improved resistance strategy (van der Salm et al. 1994).

Mechanical damage of leaves results in the transcriptional activation of a certain set of genes whose function might be to prevent further tissue damage and to inhibit the causative agent of the mechanical damage. In potato and tomato plants inhibitors of serine-, thiol, aspartate- and metalloproteinases accumulate in the plants foliage upon wounding (reviewed in Sanchez-Serrano et al. 1993). These have been postulated to interfere with efficient nutrient uptake of insect larvae feeding on leaves. Johnson et al. (1989) were able to show that over-expressing the proteinase inhibitor II (*pin* II), a powerful inhibitor of trypsin and chymotrypsin, in transgenic tobacco plants strongly affected the growth of *Manduca sexta* larvae. Also, expressing cowpea trypsin inhibitor in transgenic tobacco plants conferred increased resistance against predation by *Heliothis virescens* larvae (Hilder et al. 1987). Corn cystatin, a cysteine proteinase inhibitor, may also be a good tool for controlling insect pests as corn cystatin isolated from transgenic rice plants exhibited strong inhibitory activity against gut proteinases of *Sitophilus zeamais* (Irie et al. 1996).

Resistance strategies involving transfer of a single trait into susceptible plants must in general be viewed critically as the pathogen will easily overcome the resistance mechanism. Managing strategies to avoid these problems have been addressed by Chrispeels and Sonnewald (1997).



### β) Viral Resistance

Studying resistance against viruses has employed two main strategies: (1) relying on viral sequences and (2) based on non-viral sequences. Disease resistance or tolerance has been developed by expressing sequences derived from viral genomes in innumerable transgenic plants. The resistance through viral genes is generally referred to as pathogen-derived (PD) resistance (for recent reviews see Fitchen and Beachy 1993; Wilson 1993; Lomonossoff 1995, Baulcombe 1996). The mechanisms underlying PD can be broadly classified into those that are dependent on gene silencing events and those that are not (Baulcombe 1996). The latter may involve viral proteins, such as coat protein (CP) and movement protein (MP), a replicase or is based on viral RNA/DNA molecules. In all these cases it has been postulated that at least part of the resistance is mediated by interference of these molecules with the normal viral infection process. Thus, the transgenic coat protein has been suggested to possibly inhibit virion disassembly in the initially infected cells; the transgenic movement protein, when it is dysfunctional, could bind to plasmodesmal binding sites and thus act as competitor for the functional movement protein, or the transgenic RNA/DNA molecules could attract host- or viral encoded functions which then would be less available for interacting with the viral genome for efficient replication and spread of the virus (for references see Baulcombe 1996). A general drawback of PD-derived resistance (except for movement protein-mediated resistance) is that protection of plants is displayed only against closely related viral strains. Additional disadvantages are the possibilities of recombination events between an invading virus and the transgene, or in case of transgenic coat protein the transencapsidation of viral genomes. Therefore strategies involving non-viral sequences as transgene might be preferred.

Approaches based on interfering with processes common to multiple plant viruses have been envisaged. These include expression of ribosome-inactivating proteins (RIPs) and ribonucleases specific for double stranded-RNA molecules (Table 3). RIPs inactivate eukaryotic ribosomes by deglycosylating a specific base in the 28S rRNA. As a consequence, elongation factor 2, which is necessary for the translocation of mRNA during polypeptide synthesis, can no longer bind to the ribosomal complex resulting in inhibition of translation. In general RIPs do not inhibit "self" ribosomes. Expression of an RIP from *Phytolacca americana* (pokeweed) in cell walls of transgenic potato and tobacco plants resulted in resistance to infection by different viruses (Lodge et al. 1993). However, plants expressing high levels of pokeweed RIP were sterile. Partial resistance against tomato mosaic virus, cucumber mosaic virus and potato virus Y was obtained in transgenic tobacco plants expressing yeast-derived *pac 1* gene encoding a double-strand specific RNase (Watanabe

et al. 1995). A novel strategy employing transgenes also not derived from viruses has been investigated in transgenic tobacco plants (Mitra et al. 1996; Ogawa et al. 1996; see also Table 3). Components of the interferon-induced 2',5'antiviral system of mammals consisting of a 2',5'-oligoadenylate synthase (2-5Aase) and ribonuclease L (RNase L) were constitutively expressed. The mechanism as elucidated in mammals consists of 2-5Aase adding adenosine residues, via 2',5'linkage, to the ends of double-stranded RNA molecules (replication intermediates of single-stranded RNA viruses). The oligoadenylated, double-stranded RNA activates RNase L which hydrolyses cellular and viral RNA. The use of this system appears to be promising as complete or partial resistance was obtained against members of diverse groups of viruses: cucumber mosaic virus (Ogawa et al. 1996), alfalfa mosaic virus, tobacco mosaic virus and tobacco etch virus (Mitra et al. 1996). Yet, plants infected with potato virus Y strain T died within 20 days (Ogawa et al. 1996). The authors suggest that in this case efficient resistance was possibly not obtained due to insufficient amounts of double-stranded RNA to activate RNase L.

With the possibility to produce functional antibodies in plants (Hiatt et al. 1989) these have been considered another promising tool not only to study hormonal action (see Sect. 5.c) but also for plant disease control (Hiatt 1990). By expressing a single-chain Fv antibody directed against the coat protein of artichoke mottled crinkle virus (AMCV), plants were protected from attack by AMCV (Tavladoraki et al. 1993).

#### y) Bacterial and Fungal Resistance

By comparing plant cultivars, mainly in vegetative tissue, reacting either resistantly or susceptibly to a certain pathogen, and by applying compounds such as SA or elicitors to induce resistant responses, many genes have been identified which either were specific for the resistant situation or, more commonly, that had a faster induction kinetics in the resistant interaction compared with the situation when the plant developed disease. Many different defence-related genes were found by these approaches (reviewed by Collinge and Slusarenko 1987). A number of genes like PAL, CHS and peroxidases were involved in secondary metabolism, such as in the biosynthesis of phytoalexins or in lignification and modification of cell walls. Others encoded so-called pathogenesis-related proteins (PR-proteins) of which two classes possess chitinase and  $\beta$ -1,3-glucanase activities with in vitro antifungal effects. Other PR proteins are thaumatin-like proteins, the zeamatin family and the thionins (for references see Carmona et al. 1993). Many of these PR-protein encoding genes have been ectopically expressed and analysed with respect to their resistance characteristics (for references see Jach et al. 1995; Zhu

et al. 1996). The outcome of these experiments varied considerably, with generally enhanced resistance obtained if combinations of different PR-protein genes were expressed (Jach et al. 1995). The combinational approach not only is more effective but also offers the advantage of hopefully giving a longer-lasting protection. Another approach to isolate relevant genes conferring protection has been to extract plant compounds with antifungal or antibacterial activities from seeds to study the survival strategy of the latter in a hostile world. By this, for example, small cysteine-rich proteins were isolated from radish seeds and shown to confer enhanced resistance against the foliar pathogen *Alternaria longipes* upon constitutive expression in leaves (Terras et al. 1995). In one case, increased biosynthesis of a phytoalexin has been reported to result in enhanced fungal resistance (Hain et al. 1993). Ectopic expression of grapevine stilbene synthase in tobacco plants resulted in biosynthesis of the phytoalexin resveratrol normally not encountered in tobacco plants. These plants showed enhanced resistance to infection by *Botrytis cinerea* (Hain et al. 1993).

Plant cultivars resistant to their viral, fungal and bacterial pathogens and nematodes have been exploited for decades in breeding disease-resistant crop species. At the genetic level resistance has been attributed to single loci in many cases. However, resistance only ensues if a plant resistance gene (R-gene) meets a corresponding avirulence gene (avr-gene) on the pathogen side (incompatible reaction). The incompatible type of reaction is often accompanied by rapid cell death (necroses) at the site of infection, referred to as a hypersensitive response (HR) preventing further infection of the pathogen. In recent years, much progress has been made in cloning plant R-genes (reviewed in Bent 1996) and it has become conceivable to transfer single R-genes from a resistant to a susceptible cultivar or even a different plant species. Thus, it could be shown that transfer of the tomato *Pto* gene which confers resistance to *Pseudomonas syringae* pv. *tomato* (*Pst*) expressing *avrPto* to tobacco plants resulted in tobacco plants resistant to *Pst* (Rommens et al. 1995, Thilmony et al. 1995). The expression of the tobacco N gene which confers resistance to tobacco mosaic virus (TMV) was introduced into tomato plants, thereby conferring resistance against TMV in tomato (Whitham et al. 1996). However, transfer of a single R-gene results only in highly specific resistance limited to pathogens matching this R-gene. A much broader type of resistance can be obtained if matching pairs of R-genes *avr*-genes are ectopically expressed in a highly regulated manner (reviewed in Bent 1996; Crute and Pink 1996). This requires that downstream events to the initial recognition of the gene pair is conserved among different plant cultivars and species. It has already been shown that expression of an *avr*-gene (*avr9* from the fungus *Cladosporium fulvum*) in the cell wall of a tomato cultivar containing the corresponding R-gene (*cf*) resulted in cell death within 3 weeks after seed

planting (Hammond-Kosack et al. 1994). Thus, for instance, expressing the *avr9/cf* matching pair behind a fast pathogen-inducible promoter would allow the switching on of the HR, possibly limiting spread of the unrelated invading pathogen.

## δ) Nematode Resistance

Nematodes invade roots and cause the formation of giant cells from which the growing nematode draws the food supplied by photosynthesis in leaves. They thereby not only cause damage to roots but also limit yield of crops to a large extent. Resistance against nematodes may be obtained if feeding of the nematode or development of the giant cell is inhibited. Thus, partial resistance against *Globodera pallida* has been obtained by expressing a cysteine proteinase inhibitor from rice, oryzacystatin, in hairy roots of tomato plants, possibly affecting feeding efficiency of the nematode (Urwin et al. 1995). Alternative approaches have been to produce monoclonal antibodies specific to proteins essential for pathogenesis. Thus, single chain Fv antibodies have been produced against stylet secretions of nematodes (Baum et al. 1996, Rosso et al. 1996). So far, no beneficial effect has been observed in transgenic plants possibly due to the accumulation of the antibodies in the apoplast whereas stylet secretions appear to be injected in the cytoplasm (Baum et al. 1996).

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## Secondary Plant Substances: Monoterpenes

By Horst-Robert Schütte

### 1. Introduction

The monoterpenes are the  $C_{10}$  representatives of the terpenoid family of natural products and they diverge from higher isoprenoid biosynthesis at the level of geranyl pyrophosphate. The vast majority of the several hundred naturally occurring monoterpenes are cyclic, primarily cyclohexanoid, and they represent a relatively small number of skeletal themes multiplied by a very large range of simple derivatives, positional isomers, and stereochemical variants (Figs. 1, 2, Dev et al. 1982; Glasby 1982; Connolly and Hill 1992, Buckingham 1994). The formation of significant quantities of monoterpenes ( $> 0.1\%$  fresh tissue weight) appears to be confined to some 50 families of higher plants in which the monoterpenes are most familiar as components of the essential oils that are synthesized and accumulated in various types of distinct and highly specialized secretory structures. The chemistry and biochemistry of monoterpenoids is periodically reviewed (Banthorpe and Branch 1985; Croteau 1987; Gershenzon and Croteau 1990; Beale 1991; Grayson 1992, 1994, 1996; Dewick 1995; Chappell 1995; McGarvey and Croteau 1995). The last review in this series was published 12 years ago (Schütte 1984). The monoterpenoids show numerous kinds of ecological interactions (Harborne 1991; Langenheilm 1994).

### 2. Geranyl Pyrophosphate

Isopentenyl pyrophosphate (Fig 3. 28) is the basic  $C_5$  building block that is added to prenyl pyrophosphate cosubstrates to form longer chains. Isopentenyl pyrophosphate itself is insufficiently reactive to undergo ionization to initiate the condensation to higher terpenoids. Therefore, first it is isomerized to the allyl ester dimethylallyl pyrophosphate (26) by isopentenyl pyrophosphate isomerase. This enzyme, which has been described in plants (Spurgeon et al. 1984; Dogbo and Camara 1987; Lüt-zow and Beyer 1988), requires a divalent metal ion. Based on characteri-

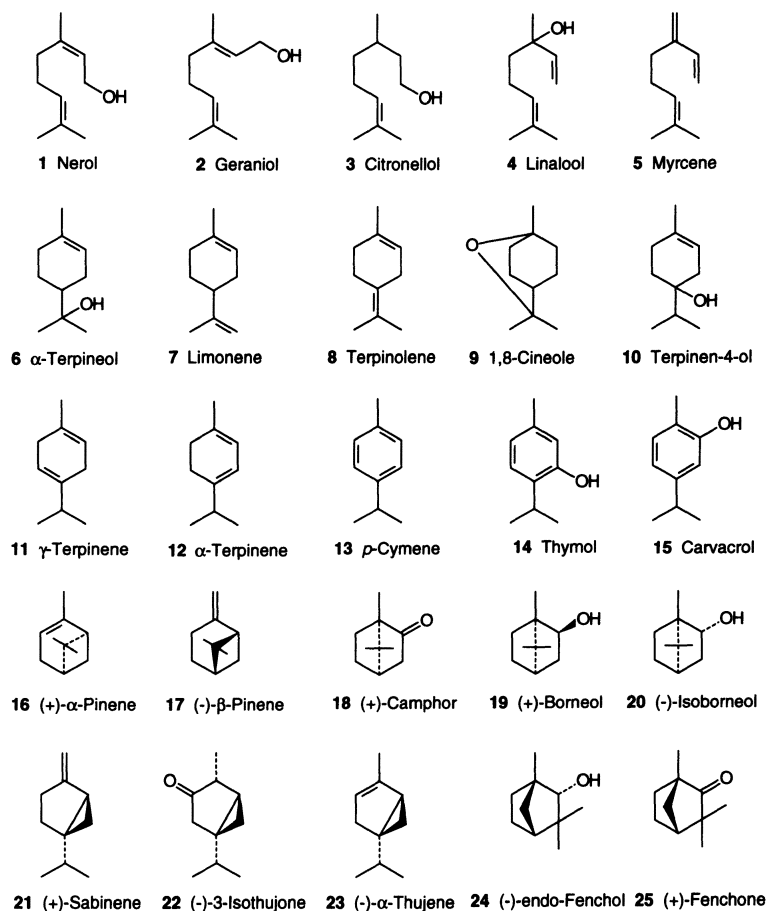


Fig. 1. Representative monoterpenes

zation of the animal and yeast enzymes, isopentenyl pyrophosphate isomerase operates through an unusual carbocationic mechanism.

Isoprene (27), the simplest of the terpenoids, is synthesized directly from dimethylallyl pyrophosphate by diphosphate elimination. The reaction is catalyzed by the enzyme isoprene synthase, which has been studied in aspen and velvet beans (Silver and Fall 1991; Kuzma and Fall 1993) and is probably localized in the chloroplasts (Mgaloblishvili et al. 1978, 1981). Isoprene is produced and emitted by the leaves of many plants and accounts for a significant proportion of atmospheric hydrocarbons (Zimmermann et al. 1978).

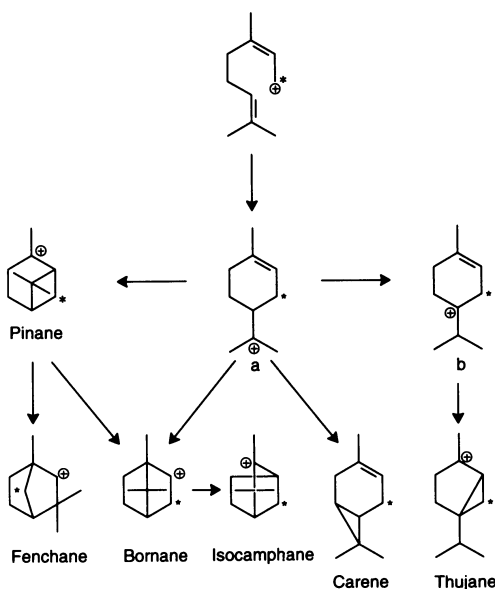


Fig. 2. Different monoterpene skeletons derived from a common precursor

The developmental regulation of isoprene emission has been examined with velvet bean leaves (Grinspoon et al. 1991). Both isoprene emission and isoprene synthase activity increase over 100-fold as leaves develop, peaking at 14 days after leaf emergence and declining thereafter. These correlative results strongly suggest that the level of isoprene synthase in the leaf is a principal determinant of isoprene production during development.

Geranyl pyrophosphate synthase forms the  $C_{10}$  intermediate from dimethylallyl pyrophosphate (26) and isopentenyl pyrophosphate (28). This synthase has been characterized in a number of plant species e.g. *Lithospermum erythrorhizon*, *Salvia officinalis* or in plastids from *Vitis vinifera* cell suspensions and is clearly separable from farnesyl pyrophosphate synthase (Croteau and Purkett 1989; Heide and Berger 1989; Feron et al. 1990; Suga and Endo 1991; Endo and Suga 1992; Soler et al. 1992; Clastre et al. 1993). The enzyme requires  $Mn^{2+}$  and  $Mg^{2+}$ . Typical are the reactions of the monoterpene cyclases, in which the required divalent metal ion is thought to assist in the initial ionization of the geranyl pyrophosphate (30) substrate leading to isomerization to the enzyme-bound intermediate linalyl pyrophosphate (31, 32), the tertiary allylic isomer. This preliminary isomerization step is necessary because the *trans*-2,3-double bond of the geranyl precursor prevents direct cyclization.



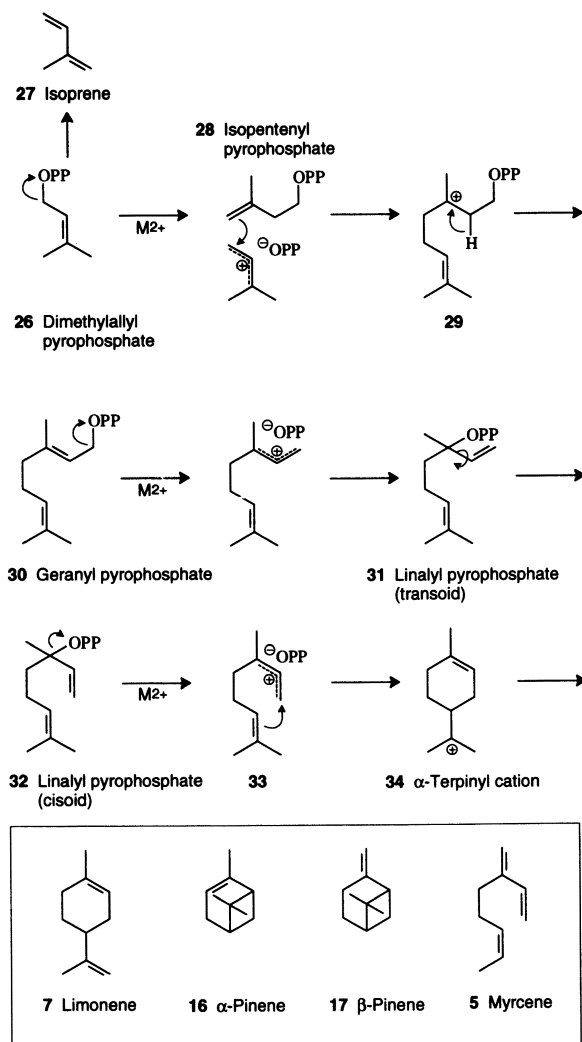


Fig. 3. Enzyme mechanism of two representative syntheses

Ionization of linalyl pyrophosphate promotes cyclization to the terminal double bond to yield the enzyme-bound  $\alpha$ -terpinyl cation (34), a universal intermediate of these cyclization reactions. The different basic monoterpene skeletons are then delineated from this highly reactive intermediate by processes that may involve internal electrophilic addition to the remaining double bond, hydride shifts, and other rearrange-

ments before termination of the reaction sequence by proton loss or capture of the carbocation by a nucleophile such as water or the original pyrophosphate anion (Fig. 2). An unusual feature of the monoterpene cyclases is that many of these enzymes produce multiple products (Wagschal et al. 1991). For example, limonene synthase, in addition to the principal cyclic product limonene (Fig. 3, 7), also generates smaller amounts of myrcene (5) and  $\alpha$ - and  $\beta$ -pinene (16, 17).

Subsequent transformations of the basic parent skeletons involve oxidations, reductions, and isomerizations to form the myriad of different terpenoids. Many of the hydroxylations or epoxidations involved are performed by cytochrome P450 mixed function oxidases (Donaldson and Luster 1991; Mihaliak et al. 1993; Bolwell et al. 1994).

### 3. Regulation

Terpenoid biosynthesis may be delimited by physical structures at histological, cellular, and subcellular levels. Where large amounts of hydrophobic terpenoids are produced and accumulated, specialized secretory structures are usually required. Common among the conifers are systems of resin ducts and blisters, and the relative complexity of these structures closely parallels their potential for monoterpene production. In conifer species, such as *Thuja plicata* (Western red cedar), which contain only scattered resin cells, levels of endogenous monoterpenes are low, as are monoterpene cyclase activities. In species with correspondingly more complex secretory structures, from resin blisters of *Abies* (fir) species to resin passages in *Picea* (spruce) and resin ducts of *Pinus* (pine), the corresponding levels of endogenous monoterpenes and monoterpene cyclase increases correlatively with the degree of specialization.

In angiosperms that produce high levels of monoterpenes, sesquiterpenes, or diterpenes, the biosynthetic machinery is often sequestered in specialized glandular structures. In *Mentha* (mint) species, for example, monoterpenes are produced primarily in the glandular trichomes of the leaf (Gershenzon et al. 1989). These trichomes are modified epidermal hairs consisting of a cluster of secretory cells, with an underlying stalk and basal cell, surmounted by a droplet of oil enclosed by the cuticle layer. Biosynthesis of other terpenoids is often restricted to specific tissues at their sites of utilization, for example, linalool (Fig. 1, 4). Biosynthesis of wound-induced monoterpenes in conifers is localized to the proximity of the wound.

Debate has raged over the subcellular localization of the early steps of terpenoid biosynthesis leading to isopentenyl pyrophosphate (Fig. 3, 28). Whereas one model holds that it is synthesized in each subcellular compartment in which it is utilized, a competing model maintains that it

is synthesized exclusively in the cytosol and is partitioned to other locations (Gray 1987). Hydroxymethylglutarate-CoA reductase, which is thought to be an important regulatory step in isopentenyl biosynthesis, has been localized to both plastids and mitochondria of radish (Bach 1987), although the *Arabidopsis* enzyme is thought to be localised only in the endoplasmic reticulum, based on the insertion of the in vitro-expressed protein into mammalian microsomes (Enjuto et al. 1994, 1995). A study with developing barley chloroplasts demonstrated that while chloroplasts from young tissue are capable of synthesizing isopentenyl pyrophosphate (28), those from mature leaf tissue rely on import of cytosolic isopentenyl pyrophosphate (Heintze et al. 1990). On the other hand, in isolated glandular trichomes of peppermint, isopentenyl pyrophosphate formation in the cytosol is blocked at hydroxymethylglutarate-CoA reductase at the time when oil accumulation is most rapid; thus, the biosynthesis of both monoterpenes and sesquiterpenes relies exclusively on partitioning of plastid-derived isopentenyl pyrophosphate (McCaskill and Crotenau 1993, 1995). It seems unlikely that a general organizational model for the origin of isopentenyl pyrophosphate can apply to all tissues and stages of development.

For monoterpenes, however, plastids are clearly implicated as the exclusive site of synthesis (Kleinig 1989). Biosynthesis of geranyl pyrophosphate (30) has been localized in plastids of *Vitis vinifera*. Isolated leucoplasts from *Citrofortunella mitis* and *Citrus unshiu* fruit are capable of monoterpene (but not sesquiterpene) biosynthesis from isopentenyl pyrophosphate (28) (Gleizes et al. 1983; Pauly et al. 1986). Isolated chromoplasts of daffodil flowers (Mettal et al. 1988) and of *Citrus sinensis* fruits (Perez et al. 1990) have a similar capacity for monoterpene formation. A study of 45 species of higher plants revealed a correlation between the levels of monoterpene biosynthesis and the presence of leucoplasts (Cheniclet and Carde 1985).

In the case of mint species, the level of monoterpene oil accumulated in oil glands over the course of leaf development appears to reflect directly the relative balance between levels of key biosynthetic enzymes (geranyl pyrophosphate synthase and limonene cyclase) and catabolic processes. When considered together, these results strongly suggest that the developmental regulation of terpenoid metabolism resides, at least in part, at the level of the first dedicated steps of biosynthesis of the various structural classes. This is not to say, however, that precursor supply is not a factor in determining the overall rates of terpenoid production.

#### 4. Linalool

The flowers of many plants emit scents to attract pollinators. The chemical structures of many floral scent compounds is known (Knudsen et al. 1993). In an investigation of the scent in the flowers of *Clarkia breweri* (Onagraceae), an annual plant which grows in the canyons of the Santa Clara mountains in the coastal range of California, it has been shown that the strong, sweet fragrance of *C. breweri* consists of at least 12 volatiles that fall into two groups, monoterpenoids and derivatives of the benzoate pathway (Raguso and Pichersky 1995).

A major component of the scent of *C. breweri* flowers is linalool (Fig. 1, 4), an acyclic monoterpene alcohol common to the floral scents of numerous other plant species. In addition to linalool, *C. breweri* flowers also synthesize and emit two linalool oxides, for which linalool is the proposed precursor (Winterhalter et al. 1986; Pichersky et al. 1994). S-linalool synthase activity is observed in *Clarkia* flower parts. This enzyme catalyzes the cation-dependent and stereoselective conversion of geranyl pyrophosphate (Fig. 3, 30) to linalool (4) (Fig. 1). S-linalool synthase is both developmentally and differentially regulated in the various floral organs. Total S-linalool synthase activity per flower was highest in petals, from which most of the linalool emission occurs. S-linalool synthase activity per fresh weight was highest in stigma and style (e.g., the pistil), but most of the linalool produced by these tissues is converted to linalool oxides by as yet unidentified enzymes.

S-linalool synthase is abundant in stigmata of freshly opened flowers, and it was purified to > 95% homogeneity (Pichersky et al. 1995). It is operationally soluble as are other monoterpene synthases, has a  $K_m$  of 0.9  $\mu\text{M}$  for geranyl pyrophosphate (Fig. 3, 30), exhibits a strict requirement for a divalent metal cofactor with a preference for  $\text{Mn}^{2+}$  ( $K_m = 45 \mu\text{M}$ ), and shows an optimal pH of 7.4. The enzyme is active as a monomer of  $76 \pm 3$  kDa. Neither S- nor R-linalyl pyrophosphates are substrates for the *C. breweri* S-linalool synthase, although this tertiary allylic pyrophosphate ester is a bound intermediate in the biosynthesis of cyclic monoterpenes from geranyl pyrophosphate in many plant species, where it also serves as an alternate substrate.

#### 5. Menthone-Type Monoterpenes

Members of the genus *Mentha* (Lamniaceae), commonly referred to as mints, are among the most well-known essential oil-producing plants (Tucker and Fairbrothers 1990). The biosynthesis of the major monoterpenes of the commercial *Mentha* species has been examined (Croteau 1991; Croteau and Gershenzon 1994). The fragrant oils of these species often contain complex mixtures of monoterpenes that are produced and

accumulated in highly specialized secretory structures, the glandular trichomes (Amelunxen et al. 1969; Gershenzon et al. 1987, 1991, 1992; McCaskill et al. 1992). A unique feature of the major type of oil gland in *Mentha* is a subcuticular cavity in which the terpenoid oils are sequestered, and which upon rupture, by physical abrasion, steam distillation, solvent extraction, etc., releases the monoterpenoid constituents responsible for the characteristic flavor and aroma of the plant.

*Mentha* oils of commercial significance include those from peppermint (*M. piperita*), American spearmint (*M. spicata*), Scotch spearmint (*M. gracilis*), cornmint (*M. arvensis*), and pennyroyal (*M. pulegium*). The characteristic components of the oils of all commercial mints, and most of their various chemoforms, are monoterpenes of either the C3-oxygenated *p*-menthane type, e.g., pulegone, (Fig. 4, 38), menthone (45), menthol (39), or the C6-oxygenated *p*-menthane type, e.g., carvone (40). All of these compounds, representing one of the largest families of cyclohexanoid monoterpenes, are formed from the common precursor (-)-limonene (7) by variations on a simple biosynthetic scheme (Kjonaas and Croteau 1983).

The obvious structural relationship between (-)-limonene (7) and (-)-carvone (40), which are major components of the spearmint oils (ca. 10 and 70%, respectively), had long suggested such a precursor-product relationship. (-)-Limonene synthase which is the major monoterpene cyclase enzyme in the mints, catalyzes a two-step reaction consisting of the isomerization of geranyl pyrophosphate (Fig. 3, 30) to enzyme-bound (+)-3S-linalyl pyrophosphate (31, 32), and the cyclization of the latter to (-)-limonene (7). The enzyme has been characterized (Alonso et al. 1992; Rajaonarivony et al. 1992a,b) and shown to be a fairly hydrophobic monomer of molecular weight of approx. 56000, with pH optimum near 6.7, and an isoelectric point of 4.35. The only cofactor required is a divalent metal ion,  $Mn^{2+}$  is preferred, and the  $K_m$  value measured for the active geranyl pyrophosphate-metal ion complex is 1.8  $\mu M$ .

Inhibition and substrate protection studies indicate that limonene synthase bears essential histidine and cysteine residues at or near the active site. An analog of the geranyl substrate, in which the terminal gem-dimethyl groups were joined to form a cyclopropyl function (6-cyclopropylidene-3E-methyl-hex-2-en-1-yl pyrophosphate), was shown to be a potent inhibitor of (-)-4S-limonene synthase from *Mentha spicata* and of several other monoterpene cyclases from diverse plant species (Croteau et al. 1993). The inhibition was concentration- and time-dependent (pseudo-first-order kinetics), as well as absolutely contingent on the presence of the divalent metal ion cofactor.

The enzyme produces primarily optically pure (-)-(4S)-limonene (Fig. 3, 7) (94%) with trace amounts of myrcene (5),  $\alpha$ -pinene (16) and  $\beta$ -pinene (17) as coproducts. Polyclonal antibodies were generated in rabbits against the denaturated limonene cyclases of spearmint, and

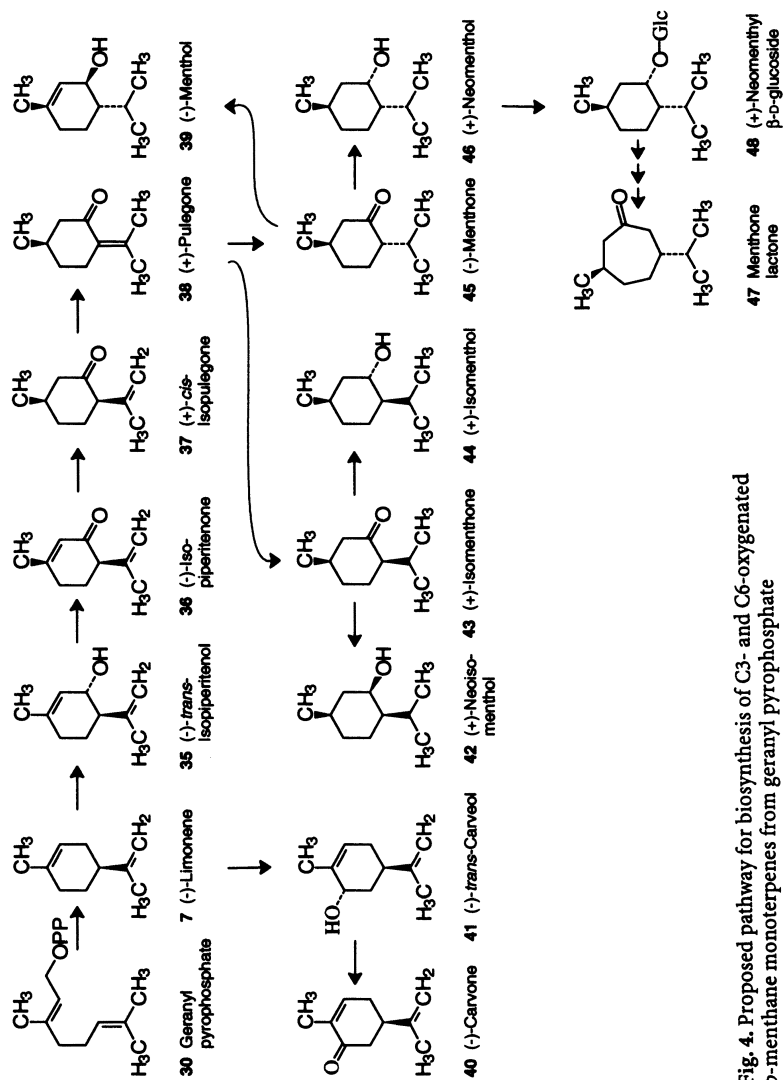


Fig. 4. Proposed pathway for biosynthesis of C3- and C6-oxygenated *p*-menthane monoterpenes from geranyl pyrophosphate

immunoblotting analysis revealed that these antibodies were very specific for the limonene synthase from all *Mentha* species tested, suggesting that this cyclase protein is very similar, if not identical, among these species (Alonso et al. 1993). The limonene cyclase is specifically localized in the leucoplasts of the oil gland secretory cells. A gene encoding limonene synthase has been isolated, sequenced, and functionally expressed in a bacterial host (Colby et al. 1993). Activity is highest in extracts of young tissue, and biosynthetic capacity progressively decreases with leaf expansion and maturity of the oil glands.

Hydroxylation is the first step following cyclization and the one that determines the oxygenation pattern of all subsequent products. These hydroxylation reactions are catalyzed by cytochrome P450 systems that utilize molecular oxygen and NADPH (Karp et al. 1990; Lupien et al. 1995). The (-)-limonene hydroxylase isolated from spearmint oil glands is located in the microsomal membranes of the secretory cells and converts this olefinic substrate to (-)-*trans*-carveol (Fig. 4, 41) as the sole product. The enzyme system has been characterized with regard to selectivity, response to specific inhibitors, and general reaction parameters. A very active, operationally soluble, NAD-dependent terpenol dehydrogenase is also present in spearmint gland extracts, that rapidly converts (-)-*trans*-carveol to (-)-carvone (Gershenzon et al. 1989; Croteau et al. 1991).

In the case of peppermint, the cytochrome P450-dependent hydroxylation of (-)-limonene (7) leads specifically to (-)-*trans*-isopiperitenol (35). The hydroxylase systems of peppermint and spearmint share many properties, including general reaction parameters, association with the microsomal fraction of the oil gland cells, and a strong preference for limonene as substrate, but the two enzymes obviously differ in the regiochemistry of the allylic oxygenation. The remaining steps of the pathway in peppermint are catalyzed by operationally soluble enzymes of the gland cells. As was the case with spearmint, the first step in the metabolism of the allylic alcohol is oxidation to the corresponding ketone, in this instance (-)-isopiperitenone (36).

The terpenol dehydrogenase from peppermint and spearmint is very similar and utilizes (-)-*trans*-isopiperitenol (35) and (-)-*cis*-carveol as substrates with roughly equal facility. The next step in the sequence is the NADPH-dependent reduction of the  $\Delta^{12}$ -double bond of isopiperitenone (36) to yield (+)-*cis*-isopulegone (37) (Croteau and Vekatachalam 1986). The reductase is highly selective for the  $\Delta^{12}$ -3-keto functional grouping and is stereospecific for reduction leading to the 1R-methyl configuration. The homoallylic double bond of *cis*-isopulegone (37) is then isomerized to the allylic position to afford (+)-pulegone (38) (Kjonaas et al. 1985). The remaining steps of the sequence involve two stereospecific double-bond reductase (NADPH-dependent) for the conversion of (+)-pulegone (38) to (-)-menthone (45) and to

(+)-isomenthone (43), and two stereospecific dehydrogenases responsible for the reduction of (-)-menthone (45) to (-)-menthol (39) and to (+)-neomenthol (46) (Kjonaas et al. 1982). The same two dehydrogenases reduce (+)-isomenthone (43) to (+)-neoisomenthol (42) and to (+)-isomenthol (44). The primary metabolites that accumulate in peppermint are (-)-menthone (45) and (-)-menthol (39).

Studies on the metabolism of exogenous (-)-menthone (45) in leaf discs suggested that the observed loss of menthone was largely the result of the selective conversion of this ketone to (+)-neomenthyl- $\beta$ - $\Delta$ -glucoside (48) (Croteau and Martinkus 1979; Croteau et al. 1984b). The glucoside is transported from leaves to roots, where it is hydrolyzed; the aglycon is oxidized back to (-)-menthone (45) and this is converted to (-)-3,4-menthone lactone (47), which after degradation is reutilized in the synthesis of different constituents of the root membranes (Croteau et al. 1984b; Croteau and Sood 1985).

Solubilized NADPH-cytochrome c reductase was purified to homogeneity from an extract of spearmint (*Mentha spicata*) glandular trichomes. The NADPH-cytochrome c reductase reconstitutes NADPH-dependent (-)-4S-limonene-6-hydroxylase activity in the presence of cytochrome P450, purified from the microsomal fraction of spearmint oil gland cells (Ponnamperuma and Croteau 1996). These characteristics establish the identity of the purified enzyme as an NADPH-cytochrome P450 reductase. It is found that cell suspension cultures of *Nicotiana tabacum* and *Catharanthus roseus* hydroxylate regio- and stereoselectively pulegone (38) and (-)-(R)-piperitone in the 4- and 6-positions (Suga et al. 1988, Hamada et al. 1994).

## 6. $\gamma$ -Terpinene

$\gamma$ -Terpinene (Fig. 1, 11) is a key component of the essential oil of thyme (*Thymus vulgaris*), as it constitutes some 30% of the oil in this species and serves as the precursor of the major aromatic monoterpenes *p*-cymene (13) (25% of the oil) and thymol (14) (40% of the oil). Cell-free extracts of thyme leaves catalyze the divalent metal ion-dependent cyclization of geranyl pyrophosphate (Fig. 3, 30) to  $\gamma$ -terpinene (Fig. 1, 11) (Poulou and Croteau 1978).  $\gamma$ -Terpinene synthase from *Thymus vulgaris* leaves was purified to apparent homogeneity (Alonso and Croteau 1991). The enzyme has a native molecular weight of  $\approx 96\,000$ . It is shown to be composed of two apparently identical subunits of  $M_r \approx 55\,000$ . The cyclase activity was inhibited by cysteine- and histidine-directed reagents. Purified  $\gamma$ -terpinene synthase also possessed the ability to cyclize geranyl pyrophosphate (Fig. 3, 30) to small amounts of  $\alpha$ -thujene (Fig. 1, 23) and to lesser quantities of myrcene (5),  $\alpha$ -terpinene (12), limonene (7), linalool (4), terpinen-4-ol (10), and  $\alpha$ -terpineol (6), all of which appear to

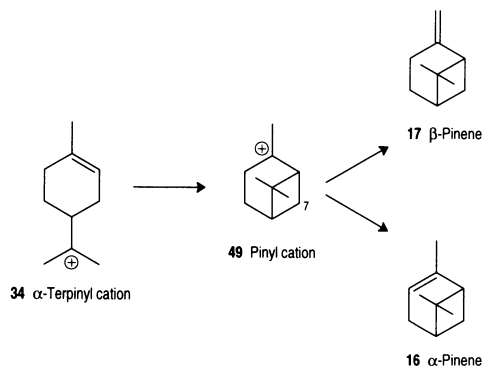


be coproducts of the reaction sequence leading to  $\gamma$ -terpinene (11). In general properties, the synthase from thyme leaves resembles other monoterpene cyclases.

## 7. Pinane-Type Monoterpenes

$\alpha$ -Pinene (16) and  $\beta$ -pinene (17) are among the most widely distributed monoterpenes in the plant kingdom and are the major constituents of the various turpentine. Studies on pinene biosynthesis in soluble enzyme preparations from common sage (*Salvia officinalis*) have demonstrated the presence of three monoterpene cyclase activities that catalyze the cyclization of geranyl pyrophosphate to stereochemically related sets of monoterpene olefins (Gambliel and Croteau 1984; Coates et al. 1987; Croteau et al. 1987b, 1988a, 1989a; Croteau and Satterwhite 1989; Wagschal et al. 1994).  $\alpha$ -Pinene (16) and  $\beta$ -pinene (17) are synthesized by the same monoterpene cyclase via deprotonation of a common intermediate (Fig. 5, 49). Pinene cyclase I ( $M_r \approx 96\,000$ ) was shown to transform geranyl pyrophosphate to the bicyclic olefins (+)- $\alpha$ -pinene (16) and (+)-camphene (Fig. 6, 52) and to lesser quantities of (+)-limonene (Fig. 1, 7), terpinolene (8), and myrcene (5), whereas cyclase II ( $M_r \approx 55\,000$ ) was shown to convert the acyclic precursor to (-)- $\alpha$ -pinene, (-)- $\beta$ -pinene, and (-)-camphene and to lesser quantities of (-)-limonene, terpinolene, and myrcene. Pinene cyclase III ( $M_r \approx 55\,000$ ) transforms geranyl pyrophosphate to (+)- $\alpha$ -pinene and (+)- $\beta$ -pinene, as well as to the monocyclic and acyclic olefins.

The general stereochemical model for the coupled isomerization and subsequent cyclization of geranyl pyrophosphate via enzyme-bound linalyl pyrophosphate (Fig. 3, 32) and the  $\alpha$ -terpinyl cation (34) involves a second electrophilic cyclization in which the remaining double bond of (34) gives rise to the pinyll cation (Fig. 5, 49) from which the indicated



**Fig. 5.** Biogenetic scheme for origin of  $\alpha$ -pinene and  $\beta$ -pinene

deprotonations lead to the pinenes (Fig. 5). During the C3-proton elimination from the pinyll cation (49) intermediate in the formation of the pinene enantiomers it was found that cyclase I and cyclase III removed the C4-proR-hydrogen of the substrate (C3-*trans*-proton to the dimethyl bridge of the pinyll nucleus) with a stereoselectivity exceeding 94% in the formation of (+)- $\alpha$ -pinene (16) (Pyun et al. 1994). Similarly, cyclase II removed the C4-proS-hydrogen of the substrate (C3-*trans* proton of the corresponding pinyll cation) with a stereoselectivity exceeding 78% in the formation of (-)- $\alpha$ -pinene (16).

(+)-Pinene synthase and (+)-bornyl pyrophosphate synthase from culinary sage (*Salvia officinalis*) have proved difficult to separate and purify. The cyclases were copurified to about 95% and labeled with the highly selective mechanism-based irreversible inactivator 6-(1-3H) cyclopropylidene-3E-methyl-hex-2-en-1-yl pyrophosphate, subjected to cleavage with bromocyanide, and the resulting covalently modified peptides were isolated (McGeedy and Croteau 1995). A radiochemical abundant 5-kDa peptide of the cleavage mixture was shown to be highly homologous, through 22 residues, to a segment (Leu197-Glu218) of (-)-4S-limonene synthase from spearmint. The result defines a presumptive active site region that bears a highly conserved sequence among these enzymes from the mint family.

Racemic thia analogs of the linalyl and  $\alpha$ -terpinyl carbocation intermediates of the reaction sequences of the pinene cyclases as well as the (4R)- and the (4S)-thia and -aza analogs of the  $\alpha$ -terpinyl cation (34) were shown to be good uncompetitive inhibitors of pinene cyclases from sage and similar enzymes, and inhibition was synergized by the presence of inorganic pyrophosphate (Croteau et al. 1986a; McGeedy et al. 1992). The noncyclizable substrate analog 6,7-dihydro geranyl pyrophosphate

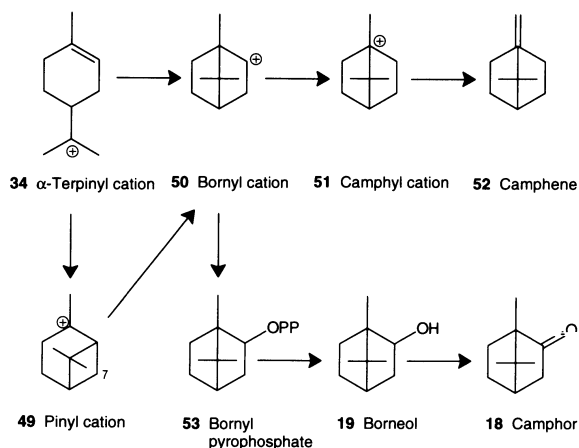


Fig. 6. A possible mechanism for biosynthesis of borneol and camphor

as well as 2,3-cyclopropyl geranyl pyrophosphate uncouple the isomerization step of the coupled isomerization-cyclization reaction (Wheeler and Croteau 1986a, 1987a,b). A partially purified and lyophilized preparation of (+)- $\alpha$ -pinene cyclase from sage was shown to convert geranyl pyrophosphate (Fig. 3, 30) to the monoterpene olefins  $\alpha$ -pinene (16), camphene (Fig. 6, 52), limonene (Fig. 3, 7) and myrcene (5) in a minimal water medium of hexane with the addition of 0.1–10% water (Wheeler and Croteau 1986b).

A major wound response in grand fir (*Abies grandis*) sapling stems is the rapid increase in monoterpene production at the site of injury (Croteau et al. 1987a; Gijzen et al. 1991; Lewinsohn et al. 1991a,b, 1992, 1993, 1994). At least six distinct cyclases, producing different monoterpene products, have been isolated; the predominant cyclase produces both  $\alpha$ - and  $\beta$ -pinene (16, 17). Antibody preparations were found to cross-react with other grand fir monoterpene cyclases that produce different olefinic products, but not with monoterpene cyclases from related conifer species (*Pinus contorta* and *P. ponderosa*) or from angiosperms (*Mentha piperita* and *M. spicata*), indicating that the wound-dependent increase in monoterpene cyclases activity is a consequence of the de novo synthesis of cyclase protein (Gijzen et al. 1992).

Two different classes of monoterpene synthases have been described based on gross enzyme properties such as metal ion requirement, pH optimum, and reactivity toward chemical modification reagents. Monoterpene synthases from various herbaceous angiosperms, which employ  $Mg^{2+}$  or  $Mn^{2+}$  as cofactors, have neutral to acidic pH optima and are inactivated in substrate-protectable manner by the histidine-directed reagent diethylpyrocarbonate, and monoterpene synthases from conifers, which cannot employ  $Mg^{2+}$  as a cofactor, require a monovalent metal ion activator, have alkaline pH optima, and are not inactivated in a substrate-protectable manner by diethylpyrocarbonate (Savage et al. 1994). Monoterpene synthase of conifers appear to have catalytically important arginyl residues specifically located at or near the active site and have at least some catalytically important thiol residues at a non-substrate-protectable region of the enzyme, in contrast to monoterpene synthases from angiosperms which appear to have catalytically important cysteinyl residues at the active site and have catalytically important arginyl residues located at a nonsubstrate-protectable region of the enzyme (Savage et al. 1995).

## 8. Bornane-Type Monoterpenes

The ketone (+)-camphor (Fig. 6, 18) is one of the major constituents of the essential oil of common sage (*Salvia officinalis*). Camphor and the related alcohol borneol (19) are commonly found together in the volatile

oil produced by a large number of plant species, and both enantiomers of each occur in nature, i.e., (+)-borneol and (+)-camphor in sage (*Salvia officinalis*) and (-)-borneol and (-)-camphor in rosemary (*Rosmarinus officinalis*). (+)Camphor constitutes nearly 30% of the monoterpenes accumulated in the leaves of sage, and as the plant approaches maturity the content decreases by roughly half. Tansy (*Tanacetum vulgare*) produces an essential oil containing the optically pure (-)-camphor as a major constituent.

The bornyl cation (50) formed both by direct cyclization of the  $\alpha$ -terpinyl cation (34) and by rearrangement of the pinyl cation (49), undergoes rearrangement to the camphyl cation (51), which yields camphene (52) upon deprotonation. Bornyl pyrophosphate cyclase from common sage (*Salvia officinalis*) catalyzes the divalent metal ion-dependent conversion of geranyl pyrophosphate (Fig. 4, 30) to (+)-(1R,4R)-bornyl pyrophosphate (Fig. 6, 53), whereas the cyclase from tansy (*Tanacetum vulgare*) catalyzes the transformation of the same acyclic precursor to (-)-(1S,4S)-bornyl pyrophosphate (Croteau and Karp 1979a; Croteau and Shaskus 1985, Croteau et al. 1986b, 1988a, 1990). Geranyl pyrophosphate was cyclized to (+)-bornyl pyrophosphate with net retention of configuration at C-1 of the acyclic precursor (Croteau et al. 1985b). A soluble enzyme preparation from sage leaves catalyzes the hydrolysis of (+)-bornyl pyrophosphate (53) to (+)-borneol (19), which is an essential step in biosynthesis of camphor (18) (Croteau and Karp 1979b; Dehal and Croteau 1987). The principal binding determinant for substrate recognition is the pyrophosphate moiety, with other domains of the substrate acting synergistically to enhance binding and to assist in aligning the allylic system and pyrophosphate in order to promote the initial ionization step of the reaction sequence (Croteau et al. 1985a; Croteau 1986; Wheeler and Croteau 1987a,b, 1988). The following formations of (+)- and (-)-camphor in this species are accomplished without loss of hydrogen from C1 of the acyclic precursor.

In mature sage leaves (+)-camphor (18) undergoes a catabolism (Fig. 7). Cell suspension cultures were shown to convert (+)camphor, in sequence, to 6-exo-hydroxycamphor (56), 6-oxocamphor (57),  $\alpha$ -campholonic acid (58), and 2-hydroxy- $\alpha$ -campholonic acid (59) (Funk et al. 1992). The first step in the metabolism of camphor is catalyzed by (+)-camphor-6-exo-hydroxylase (Funk and Croteau 1993). This enzyme is an inducible, microsomal cytochrome P450-dependent monooxygenase, which shares many characteristics with other monoterpene hydroxylases. Another catabolism via lactonization to 1,2-campholide (54) followed by conversion to the  $\beta$ - $\Delta$ -glucoside-6-O-glucose ester (55) of the corresponding hydroxy acid, 1-carboxymethyl-3-hydroxy-2,2,3-trimethylcyclomethane, which is presumed to be the transport derivative in this species, was the minor degradative pathway (Croteau et al. 1984a). The carbon from the terpenoid moiety is ultimately reutilized in

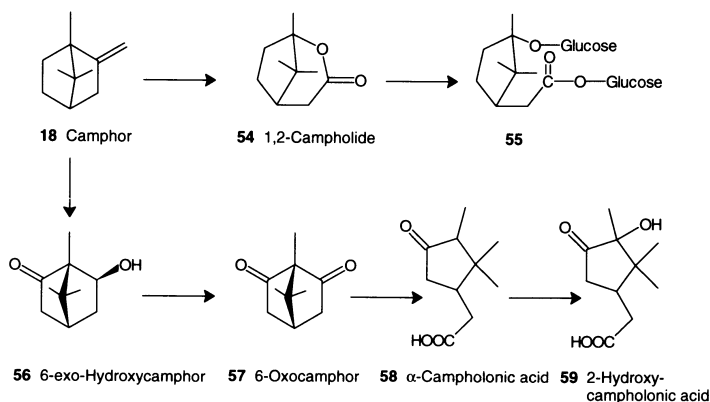


Fig. 7. Pathway for (+)-camphor metabolism in sage

the synthesis of different constituents of the root membranes (Croteau et al. 1987c). Leaves of *Salvia officinalis* accumulate monoterpenes in glandular trichomes at significant levels, whereas sage cells in suspension cultures do not (Falk et al. 1990). In vitro assay of each of the enzymes required for the synthesis of (+)-camphor in soluble extracts of the cells revealed the presence of activity. In vivo measurement of the ability to catabolize (+)-camphor in these cells indicated that degradative capability exceeded biosynthetic capacity by at least 1000-fold. Therefore, the lack of monoterpene accumulation in undifferentiated sage cultures could be attributed to a low level of biosynthetic activity coupled to a pronounced capacity for monoterpene catabolism.

## 9. Sabinene

(+)-Sabinene (Fig. 8, 21) is a major olefinic constituent of the volatile oils of immature *Salvia officinalis*, *Artemisia absinthium* and *Tanacetum vulgare* leaves, and the ketones (–)-3-isothujone (22) and (+)-3-thujone (64) are major oxygenated constituents. (+)-Sabinene is the essential precursor of these C3-oxygenated thujane monoterpenes (Karp and Croteau 1982). A soluble enzyme preparation from the leaves of sweet marjoram (*Majorana hortensis*) has been described which catalyzes the divalent cation-dependent cyclization of geranyl pyrophosphate (Fig. 3, 30) to the thujane-type alcohols (+)-(1R)-*cis*- and (+)-(1R)-*trans*-sabinene hydrate (Fig. 8, 65) (Hallahan and Croteau 1988). The basic scheme, ionization of geranyl pyrophosphate with *syn*-migration of the pyrophosphate to afford the bound tertiary allylic isomer, linalyl pyrophosphate (Fig. 3, 31), and rotation about the newly formed C2–C3 *sin*-

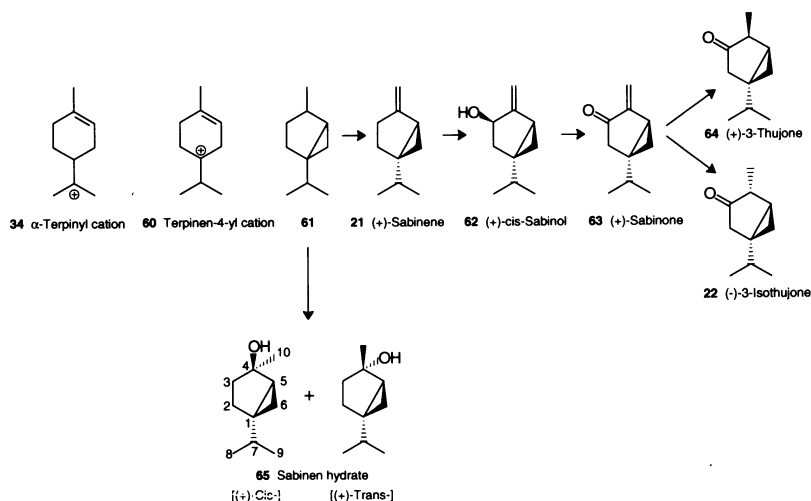


Fig. 8. Postulated pathway for origin of thujyl derivatives

gle bond to the cisoid-conformer (32) followed by a second ionization promoting the C1–C6 cyclization to afford the  $\alpha$ -terpinyl cation (34) is completed by a 1,2-hydride shift which gives the terpinen-4-yl cation (Fig. 8, 60) and subsequent internal electrophilic attack on the cyclohexene double bond generates the sabinylium (thujane) skeleton (such as 61) (Fig. 8). Capture of the resulting cation by water approaching the alternate faces of the ring produces the corresponding sabinene hydrates (65), both of which appear to be synthesized by the same enzyme. The configuration at C1 of geranyl pyrophosphate was retained in the reaction (Hallahan and Croteau 1989). A microsomal preparation from the epidermis of *Salvia officinalis* leaves catalyzed the NADPH- and  $O_2$ -dependent hydroxylation of the monoterpene olefin (+)-sabinene (21) to (+)-cis-sabinol (62) (Karp et al. 1987). The hydroxylase is highly specific for (+)-sabinene and meets most of the established criteria for a cytochrome P450-dependent mixed function oxygenase. A distinct dehydrogenase is responsible for the biosynthesis of the thujyl ketones (22 and 64) via cis-sabinol (62) (Dehal and Croteau 1987).

## 10. Carene

(+)-3-Carene (Fig. 9, 66) is a major constituent of the monoterpene fraction of many conifer oleoresins. The key reaction involving the multistep conversion of geranyl pyrophosphate (30) to (+)-3-carene is catalyzed by a monoterpene synthase. In experiments with stem disks of Douglas fir

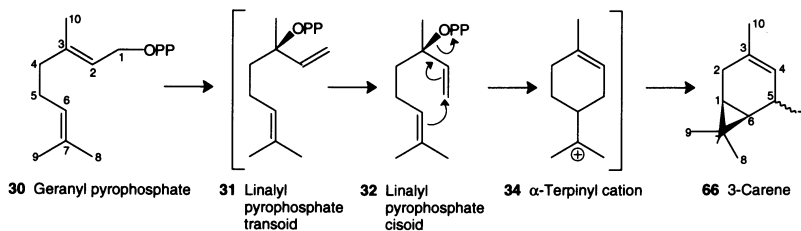
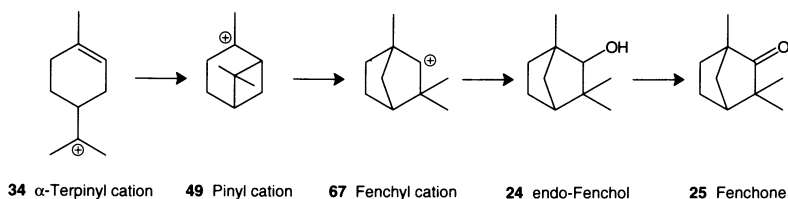


Fig. 9. Biosynthesis of 3-carene

(*Pseudotsuga menziesii*) and a partially purified preparation of (+)-3-carene synthase from lodgepole pine (*Pinus contorta*) it could be shown that during the production of (+)-3-carene the 5-proR hydrogen of geranyl pyrophosphate is eliminated during cyclopropyl ring closure (Savage and Croteau 1993). Analysis of the conformational requirements for this 1,3 elimination involving the 5-proR hydrogen suggested that cyclopropyl ring formation occurs via a  $\alpha$ -terpinyl cation (34) derived from the anti-endo cyclization of a (3S)-linalyl pyrophosphate intermediate (32) (Fig. 9).

## 11. Fenchone-Type Monoterpenes

The common herb fennel (*Foeniculum vulgare*) produces a volatile oil containing (1S)-fenchone (Fig. 10, 25) as a major monoterpene. A soluble enzyme preparation from young fennel leaves converted geranyl pyrophosphate (Fig. 3, 30) to (1S)-endo fenchol (Fig. 10, 24) in the presence of  $Mn^{2+}$ , and in the presence of pyridine nucleotide dehydrogenated the fenchol to (1S)-fenchone (25) (Croteau and Felton 1980; Croteau et al. 1980a,b). Geranyl pyrophosphate: (-)-endo-fenchol cyclase catalyzes the conversion of geranyl pyrophosphate (Fig. 3, 30) to (-)-endo-fenchol. In this conversion via linalyl pyrophosphate (32) the (3R)-enantiomer is preferred for the cyclization to (-)-(1S)-endo-fenchol (Fig. 10, 24). The isomerization step seems to be rate limiting in the coupled isomerization-cyclization of geranyl pyrophosphate to (-)-endo fenchol (Satterwhite et al. 1985). The configuration at C1 of geranyl pyrophosphate was retained in the enzymic transformation to (-)-endo-fenchol which is entirely consistent with the syn-isomerization of geranyl pyrophosphate to (3R) linalyl pyrophosphate and cyclization of the latter via the cations (49) and (67) (Fig 10; Croteau et al. 1988b). The oxygen atom of (-)-endo-fenchol is not derived from the pyrophosphate moiety of the precursor; water was the sole source of the carbinol O atom of endo-fenchol, thus indicating the participation of the solvent in terminating this presumptive carbocationic reaction (Croteau et al. 1984c, 1989b).

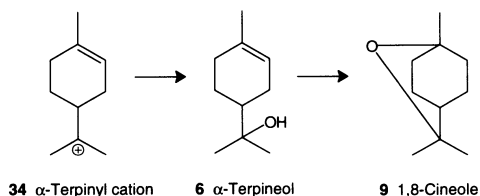


**Fig. 10.** Biosynthesis of fenchone

The isomerization component of the normally coupled reaction sequence was demonstrated directly using the substrate analog 2,3-cyclopropylgeranyl pyrophosphate and by isolating the corresponding homoallylic analog of linalyl pyrophosphate as a major reaction product. The cyclization component of the reaction sequence was effectively dissected using linalyl pyrophosphate as substrate, and both isomerization and cyclization steps were shown to take place at the same active site of the cyclase.

## 12. 1,8-Cineole

Of the symmetrical types of monoterpenoids, 1,8-cineole (Fig. 11, 9) [1,3,3-trimethyl-2-oxabicyclo(2.2.2)octane] is by far the most common, occurring in numerous essential oils. Given the relative stereochemical elements of their suprafacial isomerization to the linalyl intermediate and the anti-endo-cyclization of the latter, two possible stereochemical routes to 1,8-cineole can be formulated. These two alternative, mirror-image pathways can be distinguished by examining the fate of C1 of the geranyl substrate, through which asymmetry is introduced, and by evaluating the relative utilization of (4R)- and (3S)-linalyl pyrophosphate [and, potentially, (4R)- and (4S)- $\alpha$ -terpineol] as alternate substrate (Fig. 11). 1,8-Cineole cyclase catalyzes the conversion of geranyl pyrophosphate (Fig. 3, 30) to 1,8-cineole (Fig. 11, 9) via linalyl pyrophosphate (Fig. 3, 32) and cyclization of this bound intermediate to the  $\alpha$ -terpinyl cation (34) that is subsequently captured by water and undergoes heterocyclization to the remaining double bond (Croteau and Karp 1976, 1977, Croteau et al. 1994). The enzyme was isolated from secretory



**Fig. 11.** Biosynthesis of 1,8-cineole



cells of the glandular trichomes of *Salvia officinalis* and partially purified. As with other monoterpene cyclases of herbaceous species, cineole synthase was inhibited by cysteine- and histidine-directed reagents. Water was found to be the sole source of the ether oxygen atom of 1,8-cineole.

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## **Ecology and Vegetation Science**

## **History of Flora and Vegetation During the Quaternary**

By Burkhard Frenzel

During recent years, much interest has focused on the paleoecological conditions of High and East Asia because the monsoon system is one of the most important components of the earth's atmospheric circulation systems and influences the biosphere intensively. Also, it became clear that the relative importance of summer and winter monsoons in Asia had changed during the Quaternary repeatedly and rapidly. To better understand what might have happened and what the consequences of these repeated changes were it became necessary to investigate Quaternary paleoecology in High Asia (i.e. the Tibetan Plateau and the surrounding high mountain systems, e.g., the Tian Shan, Dzungarian Alatan, the Pamirs, the Himalayas, the Qilian Shan and the strongly elevated regions of western Sichuan) and on the Loess Plateau of East Asia. This chapter will discuss what is known at present about paleoclimatology, paleoecology and Quaternary geology in the vast regions mentioned. Necessarily, this involves the use of Chinese as well as Western literature, yet it seems that only a small part of the Chinese literature is available in Western languages. Though repeatedly interesting papers are written by Chinese colleagues in English, other important papers are only written in Chinese, yet they must not be neglected due only to the linguistic barriers. I am deeply indebted to Profs. Dr. Li Jian and Liu Shijian, Institute of Mountain Disasters and Environment, Chinese Academy of Sciences, Chengdu, People's Republic of China, for having made available several Chinese papers and for having translated them for me into English.

### **1. The Problem of Uplift of the Tibetan Plateau During the Quaternary**

Many regions of the high mountain systems bordering the Tibetan Plateau are famous for their richness in taxa of the animal and plant kingdoms and they are held to be centres of the evolution of several taxa. This is indicated by the fact that there seem to exist in various parts of these mountain systems centres of endemism. Ying et al. (1993) mapped

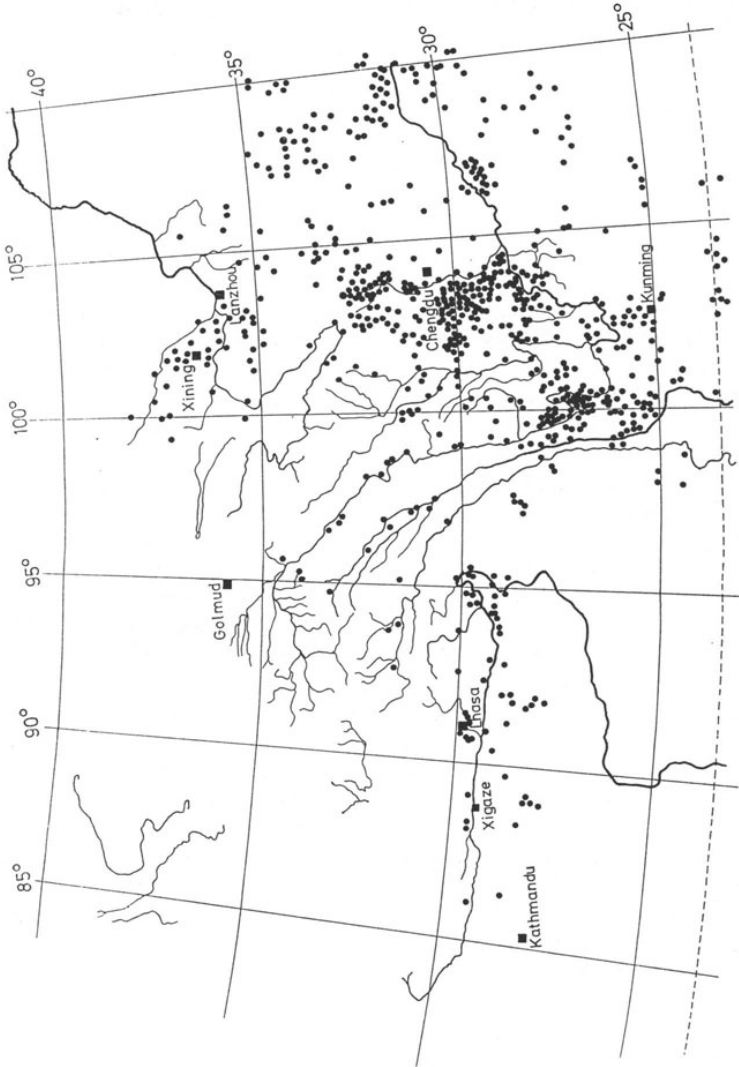


Fig. 1. Sites where endemic vascular plants are growing. (Modified after Ying et al. 1993)

where these endemic genera and species of seed plants are living. These data are compiled in Fig. 1. Though this type of investigation is strongly dependent on the intensity of botanical research in the field, it becomes evident that in the high mountains to the southwest of Chengdu and along the north-to-south-running valley of the Yangtze Djiang, just before it turns to the east, most important areas of endemic taxa are situated. Another interesting centre seems to be to the east of Lhasa, where the Brahmaputra (Yalung Tsangpo) turns to the south. This centre is illustrated in Figs. 2 and 3. The question is when these centres began to exist and what their history might have been during the Quaternary. Thus, the problem of the uplift of the Tibetan Plateau and of its bordering mountain systems becomes important for the biologist.

According to Burbank et al. (1993) there occurred about  $8 \times 10^6$  years ago within the present Indus-Ganges area a transition from moister to drier conditions. This is evidenced by changes in the  $\delta^{13}\text{C}$ -content of plant remains, pointing to a transition from prevailing  $\text{C}_3$ -plants, which were held to have been mainly trees and shrubs, to dominating  $\text{C}_4$ -plants interpreted as grasses. Though this differentiation between trees, shrubs and grasses on the basis of the  $\delta^{13}\text{C}$ -values only is not reliable, it is interesting to note that at the same time leaching of the soils seems to have increased. From this it is concluded that the monsoon climate was strengthened. At about  $7 \times 10^6$  years ago the sedimentation rate in the deep sea off the coast of Bengal decreased. This might have been caused by a reduction in the rate of uplift of the Himalayas or by vegetation becoming denser. Also, Li et al. (1979) stressed that the Tibetan Plateau

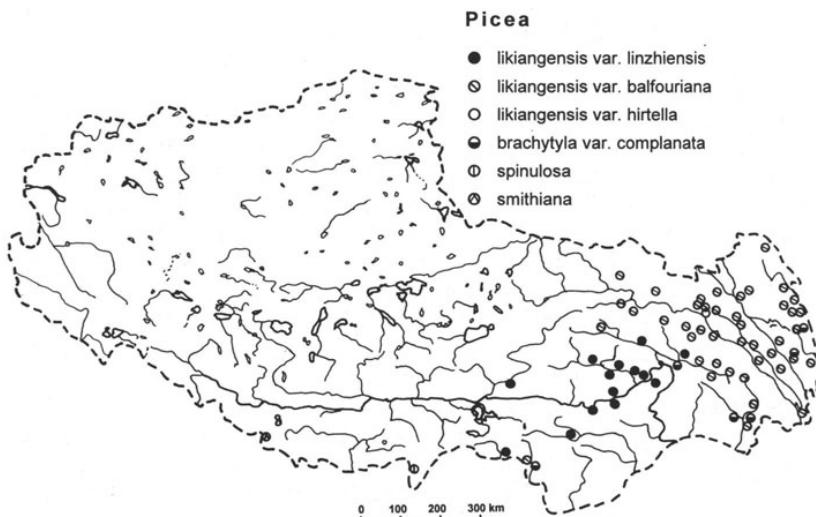


Fig. 2. Occurrences of endemic taxa of spruce (*Picea*) in Tibet. (Chen and Wang 1988)

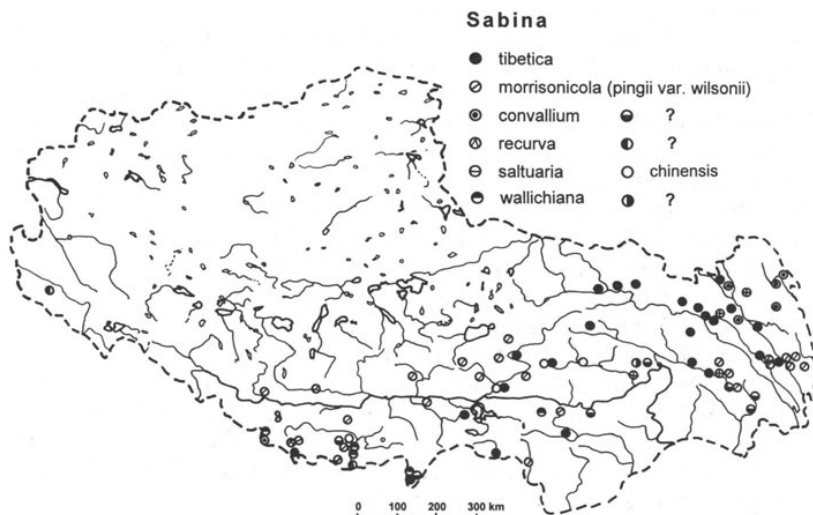


Fig. 3. Occurrences of endemic taxa of juniper tree (*Sabina*) in Tibet. (Chen and Wang 1988)

had at approximately the same time a mean elevation of about 1000 m above sea level (asl.), evidenced by planation surfaces on the Tibetan Plateau, which could be dated, on which lived a subtropical to tropical fauna. It is thought that the Himalayas and the Tibetan Plateau were intensively uplifted only about  $3.5 \times 10^6$  years ago.

For the time from  $3.5 \times 10^6$  years ago to the present, there exist two different hypotheses. One is that the uplift was slow during the Pliocene and the Early Pleistocene, speeding up most of all during the Late Pleistocene (Wang et al. 1994; Fang 1995; Li 1995; Li et al. 1979; Li and Zhu 1995; Liu 1995; Xuan and Wang 1995; Zhang and Li 1995a). It is argued that the Tibetan Plateau had reached already a mean elevation of ca. 2000–3000 m asl. by about the Middle Pleistocene (Liu 1995; Zhang and Li 1995). This would certainly mean that most of the present-day deep valleys at the southern and eastern borders of the Tibetan Plateau, in which most of the endemic taxa of the present flora are found, already existed at that time, though the valleys were not as deep and as intensively sheltered against the rain-bringing winds as they are at present. Yet the assumption that the Tibetan Plateau would have been uplifted by some hundred metres during the last 120 000 years or so is not corroborated by the configuration of the present-day and last full-glacial climatic snowlines on the Tibetan Plateau and on its bordering mountain systems (Figs. 4, 5). Thus, the other hypothesis, i.e. that the Tibetan Plateau was uplifted intensively during the Late Tertiary or at the beginning

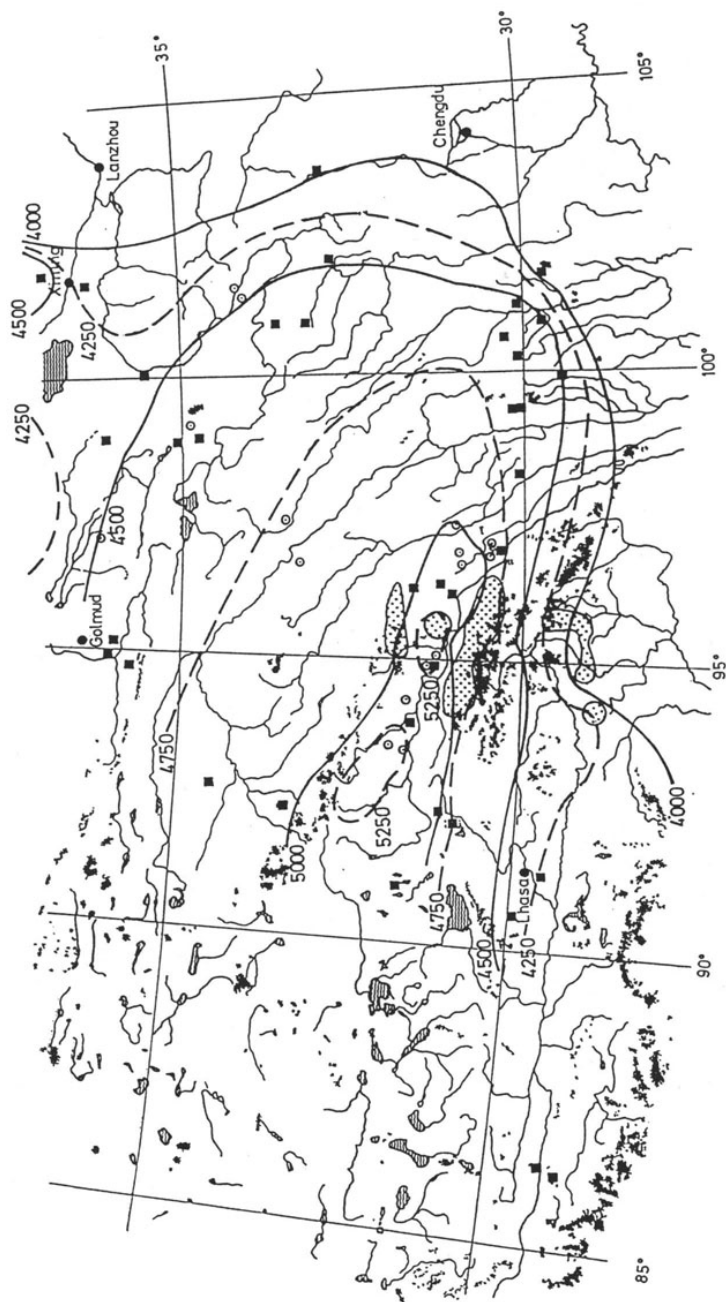


Fig. 4. Elevation (in metres) of the climatic snowline during the last glacial maximum (LGM) probably 22 000–18 000 years before present (BP; calibrated ages). (Frenzel 1994a)

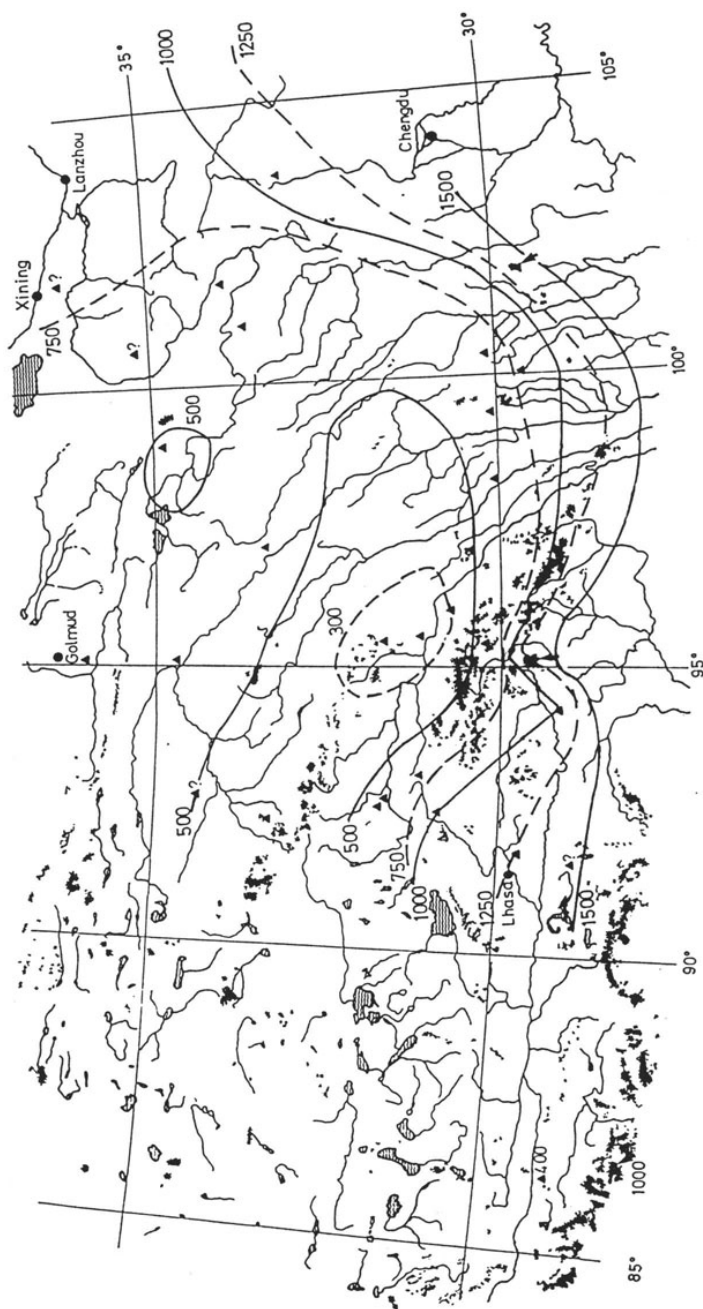


Fig. 5. Depression (in metres) of the last glacial climatic snowline compared with present-day conditions. (Frenzel 1994a)

of the Pleistocene (Fort 1996; Liu et al. 1996), seems much more reasonable.

This suggestion has two consequences: The wealth of different biotopes in the deeply incised valleys and on the extremely steep mountain slopes is much older than is thought according to the first hypothesis. This would theoretically mean that possibilities for plants to evolve in these areas would have existed there for a very long time. In this respect, it might be interesting that during the author's three expeditions to eastern and central Tibet it could repeatedly be shown that the valleys of the high mountain systems were incised already to their present-day level during approximately the Middle Pleistocene, then being filled in again by thick slope sediments. Thus, these valleys are very old. The second consequence would be that the "arid core of High Asia" is at least  $1 \times 10^6$  years old (Liu et al. 1996), pointing to a very old contrast between the phytogeographical situation within this arid core and the neighbouring much moister, yet ecologically very patchy mountain systems. Discussing these geological problems, which are so important for a profound understanding of the paleoecological evolution of central Asia, it is interesting to note that modern precise geodetic levelling is either very seldom taken into consideration, or regional differences are not accepted as such but are easily extrapolated to the whole of the Plateau (Pakhomov 1969; Li et al. 1979; Trifonov 1983; Zhu et al. 1994; Wang 1995; Zhu 1995; Fort 1996; Li and Zhu 1995). Nevertheless, when it is accepted that the deeply incised valleys in the mountain systems fringing the Tibetan Plateau are already very old, this does not necessarily mean that nowadays endemic taxa could evolve there uninterruptedly. This has to be dealt with later (see Sect. 5.c).

## 2. Climatic Change During the Quaternary

The loess plateau of northern China and the loess-covered slopes of mountain systems in Tadjikistan and Uzbekistan have become most important regions for deciphering the Pleistocene history of climate. This is due to the fact that in the regions mentioned numerous fossil soils and loess-layers were formed during the Quaternary. Magnetic susceptibility has become an important tool to correlate various loess-soil profiles of different regions with one another. The magnetic susceptibility is based on ultrafine grains of magnetite, which were formed during pedogenesis either by magnetotactic bacteria or by inorganic precipitation due to iron-reducing bacteria. The background magnetic susceptibility produced by the eolian accumulation of mineral magnetite is evidently of minor importance (Maher and Thompson 1995). On the other hand, the curves of changing magnetic susceptibility in loess-soil profiles are compared with changes in the  $\delta^{18}\text{O}$ -values of planktonic or



benthic foraminifera, which are investigated in deep-sea cores. Repeatedly, these  $\delta^{18}\text{O}$ -curves were dated by  $^{14}\text{C}$ , U/Th or magnetostratigraphy. Thus, by "wiggle-matching" the curves of magnetic susceptibility in loess and fossil soils with those of the deep-sea  $\delta^{18}\text{O}$ -curves it is tried to correlate paleoclimatic events on land with those which are documented in deep-sea sediments. Derbyshire et al. (1995) and Zhu et al. (1995) stressed that this approach involves several general problems: hiatuses in the loess-soil profiles caused by erosion may not be traced accurately; some lithostratigraphic sequences must not necessarily be complete; some fossil soils may be polygenetic; loess may have been seriously eroded by the activity of man, etc. These objections are certainly worth taking into consideration.

Sometimes it seems that the correct sequence of events is blurred by landslides. An example of this is that according to Ma et al. (1995) the high loess-profiles in the vicinity of Xining, northeastern Tibetan Plateau, contain 38 fossil soils, the sequence of which, together with that of the intercalated loesses, is correlated by the authors with deep-sea borings and with borings in the Greenland inland-ice. Yet this very region in the vicinity of Xining is according to the author's personal knowledge strongly affected by old and new landslides. The north Chinese loess-profiles are said to contain 56 (Guo et al. 1995), 27 (H.-P. Zhang et al. 1995) or 36 (Haesaerts et al. 1995) well developed fossil soils. All these profiles are held to be more or less complete, though it is argued that the Karamaidan and Chashmanigar loess profiles in Tadjikistan with 30 soil complexes are even more complete (Bronger et al. 1995).

The Chinese loesses are grouped into the oldest Wucheng loess, the middle Lishi loess and the youngest Malan loess. Each of these loesses contains some to several fossil soils. Yet the age data given for these major groups differ strongly from author to author (e.g. J.-J. Li et al. 1994; Yuan 1995; Cao et al. 1995). It is discussed whether one special complex of fossil soils, e.g. the famous  $S_3$ -soil-complex in the North Chinese Loess Plateau, is always correctly synchronized with comparable soils in other regions or other profiles in the same loess plateau (Billard and Derbyshire 1995). Thus, it is not astonishing that Guo et al. (1995) speculate about a decoupling of the Chinese loess-soil sequence from the variations in the global ice volumes, though Kukla and Cílek (1996) believe that these connections can be traced in deep-sea and loess profiles as well. On the other hand, Shackleton et al. (1995) find in the loesses of Tadjikistan and of north China good correlations to former global ice volumes, but not equally clear to the changing insolation. From the geological point of view the interrelations between loess sedimentation and phases of strengthened glacial activity appear quite convincing.

The conclusion from what has been outlined here seems to be only that during the  $2.48 \cdot 10^6$  years of the Quaternary there occurred a large number of major and minor general climatic changes which caused

considerable stress to flora and fauna, initiating long and fast migrations, and it may be that even during the Holocene the climatic boundaries of the distribution patterns of various plant and animal taxa have not been reached, since there was not enough time (Gliemerth 1995). On the other hand, one gets the impression that a complete history of climatic change during the Quaternary has not been written, yet.

### 3. Extent of the Pleistocene Glaciations on the Tibetan Plateau

To evaluate the difficulties which existed when plant and animal taxa reimmigrated to the Tibetan Plateau at the end of the Pleistocene, it is important to know whether this plateau had been covered by a huge inland-ice during the preceding glaciation or not. Kuhle (1991a,b, 1995) repeatedly stressed that the Tibetan Plateau was covered during the last glaciation by such an inland-ice, which is held to have triggered by its high reflection capacity the ensuing Nordic glaciation. Han (1995) described such an inland-ice on the Tibetan Plateau for the Early Pleistocene. The indicators for the former existence of such types of glaciation are geomorphological observations and erratics. Yet several authors deny the existence of a Pleistocene inland-ice on the Tibetan Plateau, e.g. for the whole of the plateau: Shi et al. (1990, 1992); Zheng (1989); for the Kunlun and the Karakoram: Zheng (1987); Zheng et al. (1990); Wan and Li (1992); Zhou (1994); Ono et al. (1995); Zhang and Li (1995); Lehmkuhl and Hövermann (1996); for the eastern part of the Tibetan Plateau and for west Sichuan: Li et al. (1991a,b); Frenzel et al. (1992, 1995); Frenzel (1994a); Frenzel and Liu (1994); Zheng et al. (1994); Lehmkuhl (1997); and for the Himalayas: Burbank and Cheng (1991); Li (1992); Juyal et al. (1995); Derbyshire (1996); Owen et al. (1996). In favour of this view is that clear traces of a former glaciation can only be found on the Tibetan Plateau in high mountain systems with elevations of more than 4900 m asl.; that the innumerable lakes, which are situated in the centre and the western parts of the Tibetan Plateau, are surrounded by elevated lake level beaches, which date from about 45 000 before present (B.P.) and from various parts of the last glaciation without being covered by till or other glacial sediments; that the sedimentology of the vast plains surrounding the high mountain systems does not favour the view of former inland-ice masses, and that in the eastern part of the Tibetan Plateau loess sequences are fairly widely spread, containing well developed fossil soils of evidently interglacial rank, but these loess sequences are not covered by glacial sediments (Fig. 6).

To the author it is clear that the plateau was never covered by an inland-ice. Yet there is a discussion whether larger ice-caps might have existed, e.g. in the Bayan Kara Shan or in some parts of the Tanggula Shan. According to the author's observations during three expeditions

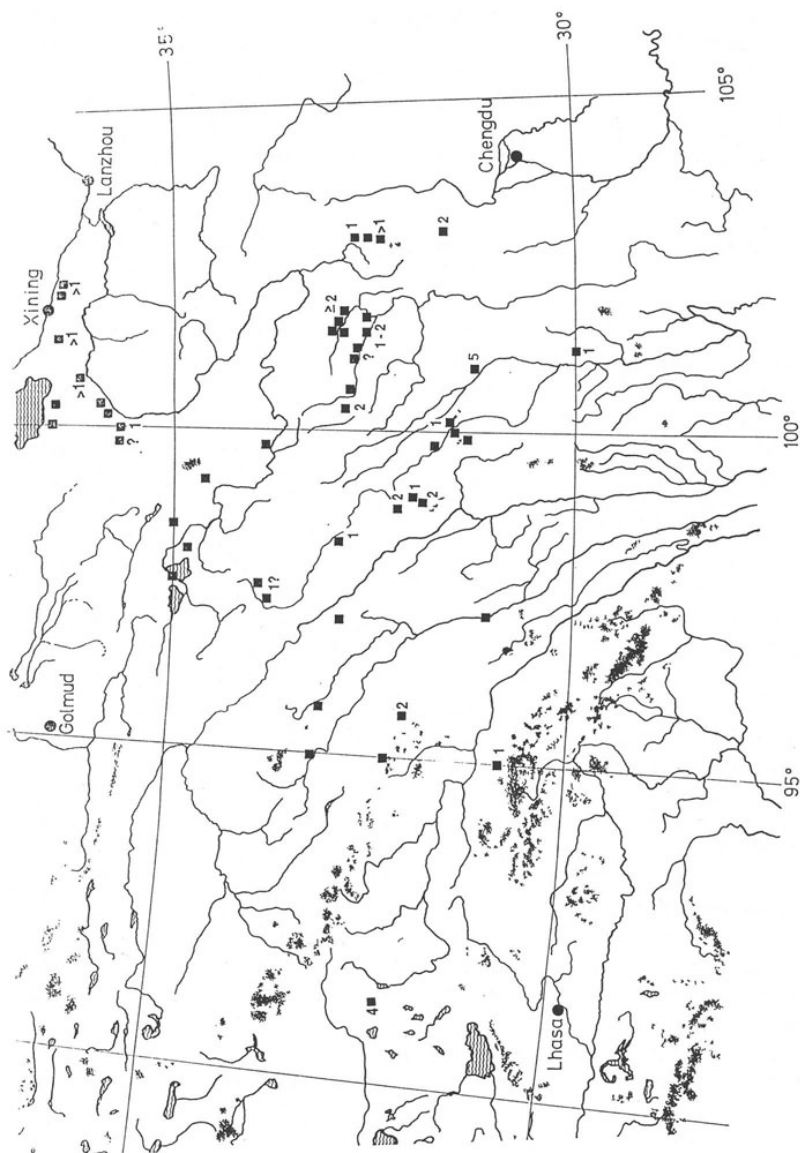


Fig. 6. Occurrence of some metre-thick loess-profiles (black quadrangles) in eastern Tibet and western Sichuan. Ciphers indicate number of well developed fossil soils per loess-profile. (Frenzel et al. 1995)

through eastern and western Tibet even these ice-caps, if they should have existed at all, were much smaller than was suggested by Li (1991a,b); Zheng et al. (1994); Zhou (1994) and perhaps by Wan and Li (1992). This means that in all probability ice could not have formed a serious obstacle for the late- and postglacial immigration of plant and animal species onto the Plateau. The extent and timing of glaciations in the mountains of eastern China are dealt with by Shi (1992) and Rost (1995).

#### **4. History of Climate During the Upper Pleistocene in Central and Eastern Asia**

CLIMAP (1981) was the first research group to map the reconstructed sea surface temperatures (SST) of the last glacial maximum (LGM), about 18 000 uncalibrated <sup>14</sup>C-years BP. Since the oceans are most important factors governing global climate, this reconstruction of former SSTs is vital for the understanding of past global climates. Yet the data given by CLIMAP did not fit to those paleoclimatic data which could be reconstructed for the Northern Hemisphere's continents (Frenzel et al. 1992). This holds most of all for the subtropical and tropical zones. From the land data one would repeatedly expect lower sea surface temperatures during the LGM. In this respect it is interesting that new investigations on the SSTs of the LGM in some of the southern oceans approach the data obtained on land. The Arabian Sea off the coast of Oman seems to have experienced SSTs at that time which were less than CLIMAP had reconstructed by about 2 or 3 °C (Emeis et al. 1995). Approximately the same holds for at least winter SSTs in the South China Sea for about 18 000 B.P.: Wu and Luo (1995); Xie et al. (1996); South and East China Seas: Lin et al. (1995); South China Sea: Huang et al. (1995). At the same time, the Tsushima Current seems to have been much stronger than it is at present (Park and Yoo 1995). These data may stimulate new paleoclimatic models to be run, if the data can be corroborated in other parts of the global oceans, too.

The Upper Quaternary begins at the onset of the last interglaciation, at about 125 000–130 000 B.P. According to the sequences of loesses and fossil soils, the Upper Quaternary was originally divided on the north Chinese Loess Plateau into the Holocene soil ( $S_0$ ), the first loesses ( $L_1$ ) and the first interglacial soil ( $S_1$ ). Yet in Europe it was shown that between the last interglacial and the Holocene there occurred several interstadials of different duration and of different paleoclimatological and paleoecological conditions. Thus, the threefold division of the younger Chinese loesses was strange. Meanwhile, it could be demonstrated that the fossil soil  $S_1$  in reality is a soil-complex, composed of three intensively weathered soils (An et al. 1991; Forman 1991; H.-M. Li et al. 1995;

Liu et al. 1995; J.-M. Sun et al. 1995; J.-Z. Sun et al. 1995a,b; Kemp et al. 1996). In other publications it is reported that this pedocomplex was composed of five soils (Chen and Li 1994; Drozdov et al. 1995), or even six soils (Fang 1995). This discrepancy is above all caused by difficulties in the differentiation between interglacial and strongly weathered interstadial soils, and Frechen (1995) is right when he states that the occurrence of forest soils in middle latitudes is no unequivocal proof of a former interglacial. Another difficulty seems to be that at present the sequence of phases of pedogenesis and loess accumulation during the Quaternary is very often dated by a comparison between curves of, e.g., total organic content in the sediments, mean magnetic susceptibility, grain size of loess particles or of quartz minerals within the loess on the one hand and  $\delta^{18}\text{O}$ -curves obtained from planktonic or benthic foraminifera in deep-sea cores on the other (see Sect. 2).

Sometimes the measured  $^{14}\text{C}$ - or thermoluminescence (TL) data are given in comparison with age data taken from the  $\delta^{18}\text{O}$ -curves of deep-sea sediments (H.-M. Li et al. 1995; Xiao et al. 1995; Guo et al. 1996). In this case, it becomes evident that there do repeatedly exist very large discrepancies between these various types of data. Frechen (1995) states that only a combination of thermoluminescence, infrared-stimulated luminescence and green-stimulated luminescence can improve the situation, if older parts of the last glaciation and the last interglacial are concerned, yet it is said that reliable data are still lacking.

Another type of uncertainty is caused by the sequence of sediments within the geological profile. For example, An et al. (1991) state that the loess-profiles they have studied on the Chinese Loess Plateau are complete without any hiatuses. Yet the author knows some of these loess-profiles personally, and all of them have hiatuses. This is already indicated by the lack of the humus-layers which should have existed on the  $B_t$ - or  $B_v$ -horizons of the interglacial soils. They have been eroded. This can easily be overlooked and can trigger the hypothesis that the transition from the interglacial or from interstadials to the ensuing glaciation or stadial was extremely fast, this being explained by Kukla et al. (1995) by the hypothesis that it could have been caused by changes in the ocean's circulation only. On the other hand, there do exist in China (Jia et al. 1995; Kemp et al. 1995a,b) and in Europe loess-profiles in which this very transition is gradual. Thus, erosion seems to have truncated several fossil soils in China and elsewhere. This may have happened synchronously with phases of a very low weathering of the loesses. In general, these phases seem to have immediately followed times of soil formation (Guo et al. 1996). It is held by the authors that climate was harsh. According to the data used (see above) these and other phases are connected with the so-called Heinrich events of the North Atlantic (Porter 1995; Guo et al. 1996; Lü et al. 1996), when suddenly huge masses of icebergs invaded this ocean. Yet if Shen et al. (1995) are right in stat-

ing that the dating of the sun-derived  $^{10}\text{Be}$ -curve in loesses in comparison with that of the  $\delta^{18}\text{O}$  in deep-sea sediments has an accuracy of about 3 000–5 000 years in the last glaciation, it becomes evident that these possible interrelations should be investigated much more intensively, i.e. by relying on an exact dating method.

Within the threefold  $S_1$ -soil-complex, one would think that the lowermost soil is the equivalent of the last interglacial proper, i.e. of the Eemian interglacial or of deep-sea stage 5e. Sometimes this soil is described indeed as the most intensively weathered one of the three fossil soils of this complex (An et al. 1991; Kemp et al. 1995a,b; Wu et al. 1995c); sometimes it is held that the three soils are of equal quality and weathering intensity (Chen and Li 1994; H.-M. Li et al. 1995; Sun 1995); or it is even thought that the two upper fossil soils are more intensively weathered than the lowermost (Sun et al. 1995a,b; Tokachi-plain in Japan: Yoshinaga (1995). Fang (1995) holds that these differences might be explained by the distance between the different loess sites and the uplifting Tibetan Plateau. On the other hand, it may be that the generally truncated soils can no more be exactly interpreted in terms of their pedogenetic intensity. In those localities, where the  $S_1$ -soil-complex contains more than three fossil soils, the uppermost are evidently divided from the lower by a relatively thick loess layer and they are less developed than the lower ones (Chen and Li 1994; H.-M. Li et al. 1995; the Krasnoyarsk region in central Siberia: Drozdov et al. 1995). Thus, it should be suggested that these upper soils were formed after the last interglacial and the initial warm-climate phases of the last glaciation, i.e. after the equivalents of the Eemian interglacial and the interstadials St. Germain I and II in Europe.

Repeatedly researchers tried to quantify the full-glacial climatic conditions during the formation of the  $S_1$ -soil complex or its equivalents. This is always based on comparisons of certain modern soil features and recent climate. These features are the pollenflora, the relative abundance of certain morphological types of phytoliths (Wu et al. 1995c; Lü et al. 1996), the magnetic susceptibility of ultrafine magnetites (Maher and Thompson 1995; Sun 1995), isotopic composition of fluid inclusions in soil carbonates and halites (Jiang et al. 1995; Yang et al. 1995) and soil types (Fang 1995). All these reconstructions show about 2 °C higher temperatures and approximately 150-mm higher mean annual precipitations than at present. These data fit generally very well into the data given in the paleoclimatological and paleoecological atlas of the Northern Hemisphere for the phases of optimal climate of the last interglacial (Frenzel et al. 1992).

The only clear contrast is produced by the biome model of Harrison et al. (1995) in which it is suggested that though in the middle latitudes of Eurasia the summer temperatures were higher by about 6–8 °C than they are at present, the winter temperatures were lower by approxi-

mately 2–3 °C. Yet the distribution patterns of various major physiognomic plant "communities" of this model differ from those which can be reconstructed by the fossil material, too (e.g. Frenzel 1968a,b).

The middle part of the last-glacial loess-soil sequence in north China is characterized by a group of faint fossil soils. They are held to have been formed between ca. 60 000 and 29 500 B. P. (H.-M. Li et al. 1995) or between about 50 000 and 20 000 B.P. (Sun et al. 1995; Liu et al. 1995; Guo et al. 1996; probably Sun et al. 1995b) and in middle Siberia, the Krasnoyarsk region, between about 34 260 and 24 000 B.P. (Drozdov et al. 1995). Repeatedly, this soil complex seems to be only very faintly developed (e.g. Kemp et al. 1996), provided that these soils were not too intensively eroded. However, this time is remarkable since it seems to correlate with a complicated phase of climatic change on the Tibetan Plateau.

Yet, discussing the history of vegetation and climate of the time mentioned, it must be taken into consideration that repeatedly serious uncertainties in the dating quality may occur. In the arid regions, like central Asia, they are caused above all by a strong solution of old isotopically "dead" carbonates in the waters of lakes and rivers. This increases the  $^{14}\text{C}$ -ages in comparison with reality. Fontes et al. (1996) found that in northwestern Tibet  $^{14}\text{C}$ -ages of these waters may be too old by about 3200 or even 5700 years and according to Yang et al. (1995) the  $^{230}\text{Th}$ -ages of halites in the Qaidam Basin seem to approach reality much better than  $^{14}\text{C}$ -ages do (Chen et al. 1990), even if it is possible that the  $^{230}\text{Th}$ -ages are about 2500 years too old. From this it follows that the dating quality increases if per geological profile several and methodologically independent datings are done.

In contrast to the generally faintly developed fossil soils on the northern Chinese Loess Plateau (faint chernozems, Fang 1995) of the time range mentioned, on the Tibetan Plateau, in the Qaidam Basin and in the Manas Lake, Dzungaria, former lake levels testify to quite other hydrological conditions than exist there at present (Chen et al. 1990; Cao 1990; Fang 1991; Li 1994; S.-Y. Li et al. 1994; Frenzel 1994a; Yang et al. 1995; Wünnemann et al. 1995; B.-Z. Zhang et al. 1995; Frenzel et al. 1995; Rhodes et al. 1996). It is worth mentioning that the relevant data were obtained by TL- and  $^{14}\text{C}$ -dating techniques. The data coincide quite well. Thus, they should approach reality to some extent. According to transfer functions obtained from the relative content of various types of opal-phytoliths and present-day climatic conditions, it is thought (Wu et al. 1995a,c) that climate was about 1.5 °C colder than today, yet that the annual precipitation was ca. 50–150 mm higher than at present. This corresponds quite well with data compiled in Frenzel et al. (1992) for the southern part of the Northern Hemisphere, since in the lower latitudes the moisture available was evidently much higher than it is today (Frenzel 1995).

However, it may be questioned whether the paleoclimatic data obtained from the relative amount of certain types of opal-phytoliths are always a sound basis for reconstructing climate, because the transfer functions use these silicates of the present-day vegetation on the north Chinese Loess Plateau, though the natural vegetation there had disappeared long ago. The malacological investigations of Wu et al. (1995b) point in the same direction: Climate between about 23 000 and 19 000 B.P. was on this plateau warmer and above all moister than it is at present. In contradiction to what has been stated, Lehmkuhl (1995) argues that the period between 32 000 and 24 000 B.P. was in the Qaidam Basin a time of maximum cold of the last glaciation. Yet this depends on the dating quality (see above), and B.-Z. Zhang et al. (1995) stress that at the same time the salinity in the paleo-Charham Lake of the Qaidam Basin was reduced in comparison with the times before and after this period, and Yang et al. (1995) report on phases of moister and cooler climate there from 50 000–45 000, 42 000–34 000 and 28 000–19 000 B.P. with intercalated warmer and drier periods, based on the  $^{230}\text{Th}$ -dating just mentioned. If the reconstructions of a relatively warm and appreciably moister climate in Tibet between the phases of utmost cold of the last glaciation are correct, this would point to quite another type of atmospheric circulation than now. It is interesting to note that at the same time North Africa experienced an equally warm and moist climate. Thus, the picture in general seems to be correct.

In the literature several age data for the beginning of the Pleniglacial of the last glaciation are given. They differ from ca. 22 000 to ca. 19 000 B.P., probably in response to the dating quality. At that time the upper part of the so-called Malan-loess was accumulated on the Loess Plateau. It was held that this loess originated in the central Asian deserts like the Gobi and neighbouring areas. Using chemical analyses on the contents of Al, Ca, Fe, K, Mn, Si and Ti (X.-Y. Zhang et al. 1995), or in general of rare earth elements (Clarke 1995a; Yu and Zheng 1995), it can be shown that the Tibetan Plateau was of some importance, too. This was evidenced by Frenzel (1994a) and Frenzel et al. (1995) mapping the occurrence of loess on the Tibetan Plateau. In its eastern part relatively often loess layer with a thickness of at least several metres are outcropping, subdivided by intensively weathered fossil soils. These sequences are lacking in the central and western parts of the Tibetan Plateau. So the frost-bitten parts of the central and western Tibetan Plateau seem to have been sources of the north Chinese loesses, too.

In general, the time of the last glacial maximum (LGM) is unanimously described as a period of utmost cold and dryness of climate. Nikolayev and Mikhalev (1995) used  $\delta^{18}\text{O}$ -values of ice layers and of segregation ice-veins in northern Siberia to reconstruct past winter temperatures. Very cautiously it can be shown that during the LGM, winters in northern Siberia were about 10–14 °C colder than they are at present



(see Frenzel et al. 1992, too). The calculated contemporary deviations from present-day conditions range in the north Chinese Loess Plateau for mean annual temperatures from  $-7$  to  $-9$  °C (Wu et al. 1995a,c; B.-Z. Zhang et al. 1995) and for the mean annual precipitation from  $-250$  mm (Wu et al. 1995a,c) or at least  $-40$  to  $-50$  mm (Maher and Thompson 1995). The mollusc fauna indicates for the time mentioned much colder and drier conditions than prevail there at present (Wu et al. 1995b). The loess sequences contain several layers of coarser dust or fine sand. These layers are always interpreted as indicating times of stronger winter monsoons (An et al. 1991; Li and Yang 1995; Sun 1995; Sun et al. 1995a,b; Xiao et al. 1995; Porter 1995; Yoshinaga 1995; Wünnemann et al. 1995; Lü et al. 1996: inner Mongolia).

To better understand what might have happened climatologically, Fan and Chen (1994) used a Global General Atmospheric Circulation Model with the prerequisite that the Tibetan Plateau was covered during winter times of the last glaciation by much more snow than at present. They found strong climatic reactions up to the mouth of the Yangtze Jiang. Yet it seems unrealistic to suggest that during winters of the Pleniglacial, Tibet was covered by much more snow than it is nowadays, since the lowering of the condensation level by increased cold would preclude a stronger influx of moisture to the Tibetan Plateau. This is evidenced by the very small lowering of the climatic snow line (equilibrium line altitude: ELA) during full-glacial times of the last glaciation in comparison with recent conditions (Frenzel 1994a; Frenzel et al. 1995). At that time loesses were accumulated even in northern India and in Kashmir. The southernmost occurrence of loesses is situated to the south of the middle Ganga River, catchments of the Sol and Belan Rivers (Pant et al. 1995).

Until recently it was unknown whether in central and eastern Asia traces of the late-glacial changes of climate could be found. The situation has changed considerably now. After the pioneering work of An et al. (1993) concerning consequences of the Allerød and Younger Dryas oscillations on the northern Chinese Loess Plateau, which still suffered from dating difficulties, repeatedly traces of these important changes of climate were found – Loess Plateau: Chen et al. (1995), Li and Yang (1995), Wu et al. (1995a–c); northeastern China: Li (1993); East China Sea: Wang (1995); northeastern part of the Tibetan Plateau: Kelts et al. (1989), S.-J. Li et al. (1994); Western part of the Tibetan Plateau: Van Campo and Gasse (1993), Li (1994), Fontes et al. (1996), Gasse et al. (1996); Dzungaria: Rhodes et al. (1996). Here, the increase of warmth and moisture can always be traced during the Bølling-Allerød complex, followed by a new cold spell with dryness at about the Younger Dryas, which in its turn gave way to a rapid warming and a remarkable increase in moisture during the beginning of the Holocene. In general, it can be shown that the climatic conditions between ca. 10 000 and 6000 B.P. (uncalibrated

<sup>14</sup>C-years) were warmer and moister than they are at present, followed by a time of slow decrease in temperatures and of much more pronounced climatic variability, just as it is observed in other regions of the globe, too. Within the time of generally warmer and moister conditions at the beginning of the Holocene, there seem to have occurred, nevertheless, some cold and dry spells, above all at about 8000–7700 B.P. – Pamir-Alai Mountains: Nikonov et al. (1989); Thar Desert: Singhvi et al. (1995); Tibetan Plateau in general: Li et al. (1982), Wang and Fan (1987), Frenzel (1994b), Yao et al. (1995), Lehmkuhl (1997); northwestern part of the Tibetan Plateau: Van Campo and Gasse (1993), Li (1994), Fan et al. (1996), Fontes et al. (1996), Gasse et al. (1996); Dzungaria and Mongolia: Hofmann (1996), Rhodes et al. (1996); eastern part of the Tibetan Plateau: Wang (1987), Kelts et al. (1989), Chen et al. (1990), S.-J. Li et al. (1994), Yu and Kelts (1995); Tibetan Plateau and lowland China: Fang (1991), Shi et al. (1993), Li (1993), Lin and Wang (1994); East China Sea and Kurile Islands: Korotky et al. (1995), Wang (1995); Taiwan: Chen et al. (1993), Yeh et al. (1995).

Repeatedly it was calculated that during the climatic optimum, climate was warmer by about 1–1.5 °C than at present combined with a higher moisture available for the biosphere (50–100 mm). These data fit quite well into the hitherto developed picture of the climatic situation at these times (see Frenzel et al. 1992). Yet, in each case the same dating accuracy, which was developed by Björck et al. (1996) for the Younger Dryas to Preboreal transition in central and northwestern Europe, could not be reached.

The decrease in temperature just mentioned since about 6000 B.P. should have influenced the position of the upper timberline in the high mountain systems of eastern and southern Tibet. Yet, it is astonishing to note that the uppermost living trees or small stands of various tree species can even nowadays be observed in eastern and southeastern Tibet at elevations of up to 4700 m asl (Fig. 7). From this follows that the deterioration of climate cannot have been as strong as it is sometimes suggested, when "dramatic" changes of climate are mentioned in the younger part of the Holocene. It seems that grazing and cutting trees for a long time was much more effect than the indisputable cooling of climate.

The youngest history of climate can be reconstructed by historical data (Wang and Wang 1989; Wang et al. 1991, for the last 500 years) or by dendroclimatology, combined with glacier history. In the Karakoram Mountains, as well as in southeastern Tibet, vicinity of Qamdo, Kam Province, living juniper trees (genus *Sabina*) were found, the ages of which are about 1300–1500 years or even more (Bräuning 1994a,b, Esper et al. 1995). Together with dead trees standing at the same sites, relatively long dendroseries can be constructed. They show, of course, that on the eastern Tibetan Plateau different growth-climate provinces do

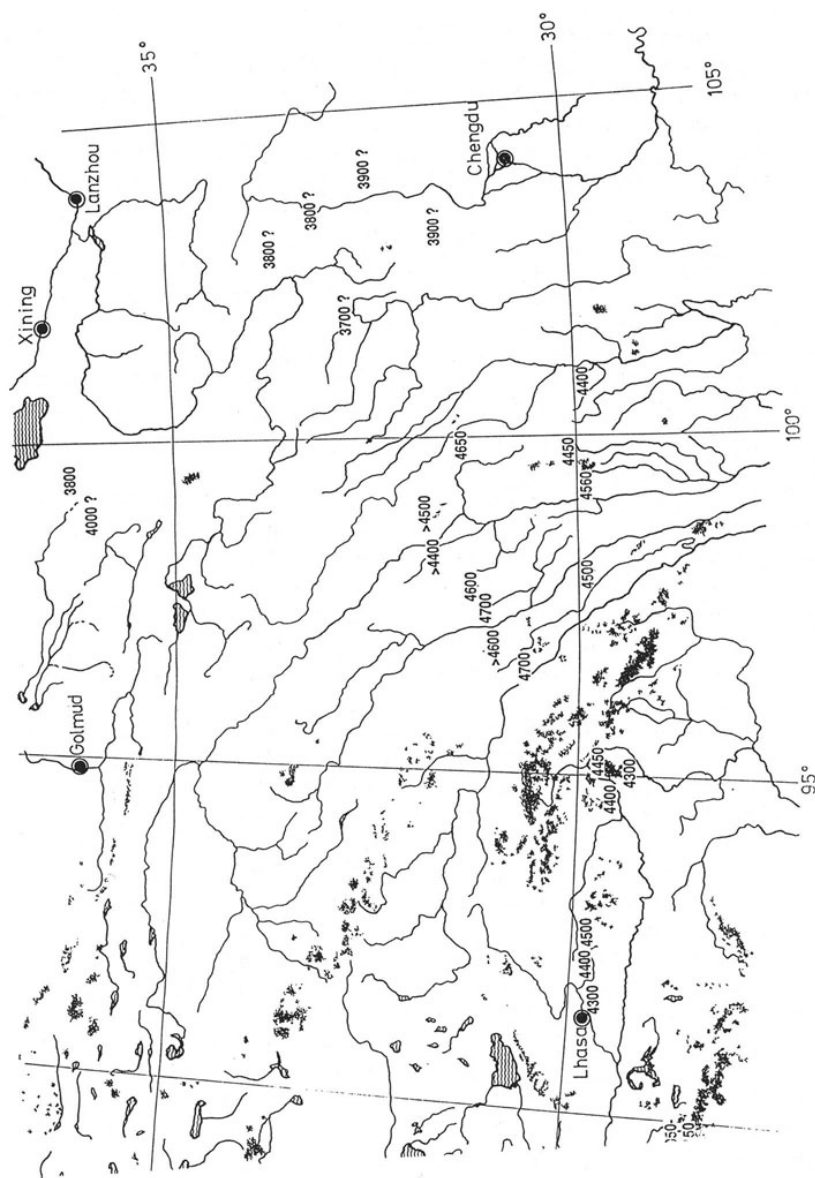


Fig. 7. Elevation (in metres) of the modern alpine tree-timerline in eastern Tibet and western Sichuan

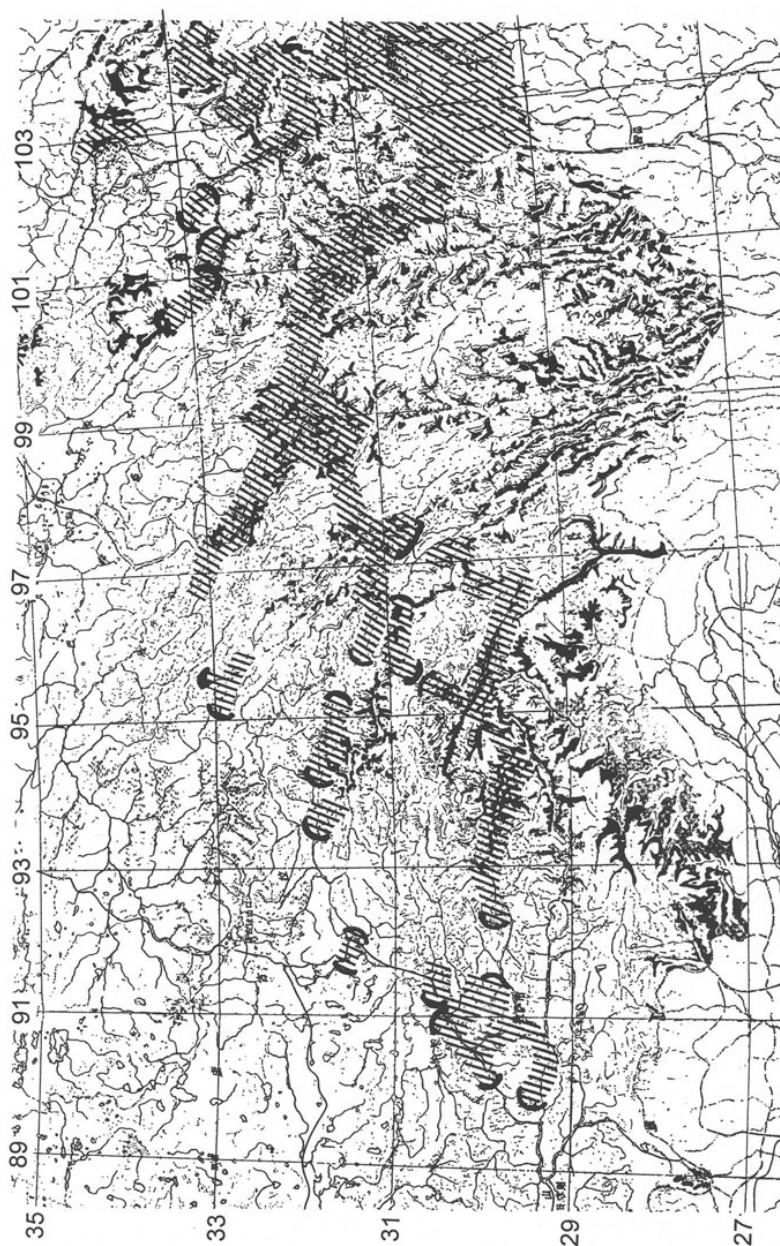


Fig. 8. Distribution pattern of present-day forests in southeastern Tibet and western Sichuan (black and grey; Tibet Atlas, 1990) and of probably mid-Holocene forests (hatched; observations during 1989, 1992 and 1996 expeditions)

occur (Bräuning 1994b); on the other hand, it becomes evident that indisputable traces of the European Medieval Climatic Optimum and of the Little Ice Age can easily be found there. Moreover, younger phases of glacier advance and retreat can be dated relatively accurately, pointing for the younger geological past to a pronounced variability of climate (Bräuning and Lehmkuhl 1996). Z.-H. Li et al. (1995) used the  $\delta^{13}\text{C}$ -values in tree rings (*Pinus tabulaeformis*) on the north Chinese Loess Plateau when studying the history of climate. Yet, since only three trees were used the results are not too convincing.

Summing up what has been said about dendrochronology, this way of research may help solve paleoclimatological/paleoecological problems which exist in vast regions of southern and southeastern Tibet, e.g. the increasing destruction of the vegetation cover in the alpine region (Miehe 1996). It is thought that this destruction is to some extent caused by indirect human activities, like grazing and trampling by the grazing animals. On the other hand, it is suggested that changes of climate might be of some significance, too, since there exists a parallelism between the destruction of the vegetation cover and southerly foehn winds. Without neglecting the strong influence of winds on the vegetation, according to the author's own field-work in Tibet, the author feels that the long-lasting influence of man and of his herds is much more important (Fig. 8).

## 5. Vegetation History

### a) General Remarks

To reconstruct the outlines of the former plant geographical setting on the north Chinese Loess Plateau, occasionally  $\delta^{13}\text{C}$ -values of secondary soil carbonates or of  $\text{C}_{\text{org}}$  are used (e.g. Han and Jiang 1995; Wang et al. 1995). The pedogenic soil carbonates are held to be governed by the  $\delta^{13}\text{C}$ -values of soil  $\text{CO}_2$ , which in its turn depends on the relative amount of  $\text{C}_3$ - and  $\text{C}_4$ -plants. On the other hand, total  $\text{C}_{\text{org}}$  is strongly influenced by the same relation. From the data obtained it is concluded that during interglacial times the Loess Plateau was in general governed by grassland. Only the second soil of the soil complex  $\text{S}_2$  seems to indicate that about 66% of the former biomass was formed by  $\text{C}_4$ -plants (Han and Jiang 1995). Yet, Wang et al. (1995) suggest that the interglacials in the Zoige Basin, northwestern Sichuan, were characterized by predominantly occurring  $\text{C}_3$ -plants. These interpretations become difficult when it is said that  $\text{C}_3$ -plants are in general trees and bushes,  $\text{C}_4$ -plants grasses, and if it is concluded from these assumptions that the Loess Plateau was in general covered by grasslands. Even at present on the Loess Plateau trees grow quite well and reafforestations work astonishingly well there

in several sites which are not confined to valleys or depressions only. A very comprehensive and competent review of the state of knowledge in Upper Quaternary vegetation history in the distribution area of present-day temperate forests in China is given by Liu (1988). Ying et al. (1993) give a comprehensive palynological description with diagnoses of the Chinese endemic genera and species of seed plants.

#### b) The Pliocene to Middle Pleistocene

A reliable reconstruction of vegetation history of the Pliocene to Middle Pleistocene is seriously hampered by difficulties in determining the exact stratigraphical position and by generally only very small quantities of sporomorphs counted per sample. Nevertheless, some general trends can be recognized.

According to Li (1994) Pliocene sediments, above all on the southern slopes of the Kunlun Mountains and in the Hoh Xil Shan, central Tibet, are characterized by a pollen flora rich in arboreal pollen with *Picea*, *Abies* and *Betula*. *Podocarpus* and *Carya* were only rarely found. It is thought that this vegetation points to warm montane conditions. On the other hand, the sediments of the Lower Pleistocene are governed by the pollen flora of forest-steppe, grassland and desert-steppe. Zhou et al. (1976) report on the stratigraphical division of the Quaternary in the northern part of the Qomolangma (Mount Everest) Massif. It is said that the Lower Pleistocene is represented by sediments of the Xixiabangma Glaciation and the Pali Interglacial, the Middle Pleistocene by the Niengqiangtanglha Glaciation and the Jiabula Interglacial, and the Late Pleistocene by the Qomolangma Glaciation, which was followed by the Holocene or the Yali Interglacial.

The Lower Pleistocene, investigated in the vicinity of Pali, 4400 m a.s.l., was characterized by prevailing nonarboreal pollen (NAP), in which *Artemisia*, Poaceae, Chenopodiaceae and several herbs dominated. The arboreal pollen was very faintly represented (*Betula* prevailing). Only the uppermost sample was characterized by 55.6% arboreal pollen (AP), with *Alnus*, *Quercus*, *Betula* and *Pinus* prevailing, together with *Abies*, *Carpinus*, *Tsuga* and *Picea*. The NAP flora is rich in various taxa. It is thought that the pollen flora reflects a broadleaved forest surrounding the former lake, with air temperatures 6–12 °C higher than at present at the beginning of the Pali Interglacial, yet the pollen quantities reported would not favour this view. The Jiabula Interglacial is described at an elevation of about 4900–5100 m a.s.l. as being characterized by AP, above all by two different types of *Picea*, with only a very small share of deciduous broadleaved trees. In this case, too, it is held that climate was much warmer, yet nothing is reported on the present-day pollen influx, which should be strongly influenced by AP via the often occurring

foehn-winds. On the other hand, it is thought that at an elevation of about 4660 m a.s.l., at the northern flank of the Xixiabangma, roughly synchronously with the vegetation just mentioned there existed a vegetation characterized by 43.7% AP, predominantly *Pinus*, whereas the NAP flora was dominated by *Artemisia* and *Ephedra*. It is not clear how reliable the stratigraphic datation is.

According to Shi (1995), *Abies* immigrated to north China (between 34.5 and 38.5 °N and 106.5 and 117 °E) at about 4 100 000 years ago, followed by *Picea* at about 2 300 000 B.P. Since approximately 2 300 000 B.P., both genera retreated, being replaced by steppe communities. During the Pliocene, the conifers mentioned were accompanied there by *Carya*, *Liquidambar*, *Podocarpus*, *Hamamelis*, *Tsuga*, *Ginkgo*, *Anacardiaceae* and *Rutaceae*. At that time *Picea* and *Abies* formed riverine forests. Later on they were replaced by cold-resistant taxa. [The Middle Pleistocene pollen flora of the Negoya and Kuisanagi formations in central Japan are described by Konomatsu and Shinohar (1995)]. The pollen-flora is divided into several pollen assemblage zones, which were in general dominated by *Fagus*, *Picea*, *Cryptomeria*, *Ulmus-Zelkova* and *Lagerstroemia*. [For short remarks on the Pliocene forest vegetation in south Tibet see Tang and Shen (1995b)]. It is stressed that by the end of the Pliocene and during the Middle Pleistocene the previously important forests of *Quercus* (oak) and *Cedrus* were replaced between the Himalayas and the Nienquientanglha Shan by deciduous broadleaved tree species and by a wealth of herbs: climate became drier as a consequence of the uplift of the Himalayas. For reconstructing history of vegetation and climate in southern Tibet during the Quaternary, Shen and Tang (1994) used principal component analyses on four Pleistocene and two Holocene pollen profiles. Eleven vegetation types are reconstructed, yet it may be questioned whether the statistical basis for this is firm enough, since in general the pollen floras reported are very poor in palynomorphs.

### c) The Upper Quaternary

The vegetation history of the Bandung Plain, west Java, about 670 m a.s.l., was comprehensively studied for the time period about 126 000–16 600 BP by Van der Kars and Dam (1995). During the last interglacial, a species-rich swamp vegetation had developed, with *Macaranga/Mallotus*, *Barringtonia* and *Lagerstroemia flos-reginae*. At the end of the interglacial, lower montane forest spread in the surroundings of the lake, with dominating *Castanopsis/Lithocarpus* and *Quercus*. This was followed between about 81 000–74 000 BP by a compression of the submontane forests and the lowering of the lower montane forest belt. Maximum development of this tendency was reached at about 47 000–

20 000 B.P., when *Myrsine*, *Dacrycarpus imbricatus* and *Dodonaea* became important elements. After 16 000 B.P. the montane vegetation began to move again to higher altitudes. It is concluded that before 16 000 B.P. temperatures must have been lowered in comparison with present-day conditions by about 7.2 °C. This fits quite well to the SST data already discussed (see Sect. 4) for the LGM in the South China Sea. Thus, it becomes evident that during the LGM even the present moist tropical zone had strongly suffered from relatively cold and dry climates.

According to pollen analyses (pollen-influx studies) the last interglacial vegetation of the central north Chinese Loess Plateau was characterized by much *Corylus*, with only some *Pinus* and broadleaved deciduous trees (Sun et al. 1995), whereas according to the same authors the vegetation of the same age in Beiyuan, Gansu, was governed by predominating AP, in which *Abies* and *Picea*, together with *Pinus*, had reached the highest values. Deciduous broadleaved trees were of minor importance (Sun et al. 1995). The last interglacial vegetation history of the Zoige Basin, northwest Sichuan, causes some stratigraphical problems (G.-X. Liu et al. 1994). The pollen profile of the 120.4-m deep boring was dated by the authors by paleomagnetic investigations, <sup>14</sup>C-datations and by calculations of the sedimentation rates. On the basis of the age data calculated by the sedimentation rates it is concluded that the last interglacial in the Zoige Basin (127 000–110 000 B.P.) was characterized by an *Artemisia* steppe community, followed by a spreading of *Abies*–*Quercus*–*Picea* forests with some *Betula* and *Castanea*. This was replaced allegedly in deep-sea stage 5c by a dense *Abies* forest, in which *Betula*, *Quercus* and even *Tsuga* were remarkable. The steppe element was only faintly developed. This interpretation causes some difficulties, because the Zoige Basin (about 3500 m asl) is even today surrounded by *Picea*–*Abies* forests, rich in *Rhododendron* and clad with lichens (*Usnea*). The lowermost forests begin at about 3600 m a.s.l., i.e. 100–150 m above the surface of the Zoige Basin. During the Holocene (see later) the region was characterized by *Picea* and *Abies* forests, with some *Quercus* and other broadleaved tree species. Some steppe elements thrived there at that time on drier habitats. Thus, the author thinks that the relatively dense *Abies* forests, reported by G.-X. Liu et al. (1994), in reality date from deep-sea stage 5e, i.e. from the last interglacial proper, yet not from deep-sea stage 5c.

Full-glacial times seem to have been characterized in the Zoige Basin by a steppe vegetation, poor in species, governed by *Artemisia*, Cyperaceae and Rosaceae [phases 7–9 of G.X. Liu et al. (1994)]. It may be that at the time, when in western and central Tibet huge lakes existed (Sect. 4), in the Zoige Basin a forest-steppe with some spruce, birch and fir had developed (phases 5 and 6 of the authors). At the same time the Loess Plateau was covered by a steppe vegetation, in which *Artemisia*, Chenopodiaceae, Asteraceae, Ranunculaceae and Cruciferae were most



prominent. The pollen influx was relatively high. Thus, the vegetation cannot have been too sparse (Sun et al. 1995) [From central Honshu, Japan, a rich macrofossil-flora is reported, dating from about 22 000–12 900 B.P. It points to species-rich subalpine forests near the present-day sea level (Noshiro 1995) and Sase et al. (1995) reconstructed for about the same time a vegetation composed of grasslands and coniferous stands in northern Japan, using phytoliths].

As already mentioned (see Sect. 4), the late-glacial sequence of climatic oscillations which is so well known from Europe and North America, can now be traced on the Tibetan Plateau, too. According to the general climatic setting the formerly prevailing desert-steppe was transformed during the Bølling-Allerød complex into various types of steppe vegetation (Van Campo and Gasse 1993; Gasse et al. 1996; Yu and Kelts 1995; Tang and Shen 1995a; Rhodes et al. 1996) and paleosoils were formed (e.g. Qui et al. 1995). On the other hand, it may be questioned whether the steppe-tundra transition at about the same time in Beringia was caused indeed by an "overkill" by the large herbivore fauna, as Zimov et al. (1995) suggested. If the author understands correctly what occurred there or elsewhere in North America and Europe, an "overkill" by early human societies should be excluded at that time.

In the introduction to this chapter it was stressed that neither the geographical position nor the species composition of the glacial refuge areas of a demanding flora and vegetation are known in the southern and eastern mountain systems. Thus, a paper of Jarvis (1993) on the vegetation history of a site in Mian Ning County, southwest Sichuan, is extremely welcome. It could be shown that by about 11 000 B.P. the area was rich in *Artemisia*, Gramineae, herbs, some pine, relatively much birch, with strong influx-values of *Sabina* pollen. Broadleaved thermophilous trees were present, yet seldom: *Ulmus*, *Tilia*, *Fraxinus*, *Carpinus*, *Celtis*, together with *Pseudotsuga* and *Cunninghamia*. This flora and vegetation was replaced there before 9100 B.P. by mesic taxa of cool-temperate climates. Thus, this region, which might have belonged to a more important refuge area, seems to have housed some of the more important tree taxa during full-glacial times. [The full- and late-glacial flora and fauna in the Lake Nojiri region, Nagano, central Japan, are described by Kondo and Sakai (1995)].

The Holocene started with a very rapid increase in moisture nearly everywhere in the region studied in this chapter. This happened at about 10 000 B.P. (uncalibrated years), though there did exist regional differences, with an earlier onset in the southern regions and some delay more to the north (Frenzel 1994b). This transition seems to have happened so fast that the impression is given that the rapidity is only pretended by hiatuses. Yet, in the Zoige Basin, northwest Sichuan, the normal sequence of invading tree taxa could be traced just as it is known from Europe at the very transition from full-glacial to late-glacial times

(Frenzel 1994b). After having established itself, the forest vegetation in the moister parts of the Tibetan Plateau remained more or less unchanged until about 5000–4000 B.P. (Chen et al. 1990; Thelaus 1992; Van Campo and Gasse 1993; Frenzel 1994b; G.-X. Liu et al. 1995; Qui et al. 1995; Andreev 1995; Song and Wang 1995; Rhodes et al. 1996; Van Campo 1996). The time when this climatically better phase ended differs from region to region (Frenzel 1994b). It cannot be denied that in the second part of the Holocene a slowly proceeding deterioration of climate had happened nearly everywhere in the Northern Hemisphere (Frenzel et al. 1992). Yet, it may be questioned how intensively it had worked. In the Zoige Basin, 3500 m a.s.l., northwest Sichuan, it can be shown that this transition was climatically not important enough to explain the strong retreat of the forest there. At about 5300–5100 B.P. parallel to the retreat of the forests, climate seems to have become at least locally moister there and the long-distance pollen floras point to a strong summer monsoon.

From this, Thelaus (1992) and Frenzel (1994b) suggested that human impact could be felt, too. In this respect, it should be mentioned that traces of prehistoric human activities are found repeatedly on the Tibetan Plateau, dating from Neolithic times (CPAM 1985; Tibet Atlas 1990; Freund 1991; G.-N. Liu et al. 1994). In the vicinity of Qamdo, southeast Tibet, Kam Province, 3600 m a.s.l., millet was grown by the population of large villages at about 4300 B.P. (uncalibrated data; CPAM 1985). On the other hand, it is strange to see repeatedly in road outcrops in the eastern part of the Tibetan Plateau fossil forest-soils, evidently dating from the Holocene, in areas which are covered by a steppe-like vegetation now, though copes of trees or solitary trees can be met with there even at present (Fig. 8). Sometimes in these fossil soils layers of charcoal are found, yet never tree-roots or stumps. Thus, it is concluded that these fossil soils testify to the natural conditions which existed there before man began to destroy these forests long before the recent past. The connections between human activities and soil and loess sequences on the Loess Plateau are outlined by Zhou (1995).

## 6. The History of Flora

As will be seen from the preceding pages, we are only at the very beginning of an understanding of vegetation history in central and eastern Asia. Nevertheless, some data are available, too, on the history of flora. Lin et al. (1995) report on the distribution pattern of *Picea* and *Abies* during the LGM in eastern China. This is shown in maps, together with those of the former distribution patterns of some last-glacial animal species. Lie et al. (1994) discuss the Middle Pleistocene distribution of *Fagus hayatae* spp. *hayatae* in Taiwan, and Huang (1988) speculates about the fact that 14.7% of the flora of vascular plants of the Qomo-

langma–Xixabangma region is endemic. It is held that this indicates the youthfulness of the flora and in its turn the rapid uplift of the mountain massif. It would perhaps be wise to differentiate between progressive and conservative endemism. Miehe (1996) concludes from various patterns of disjunctions in the Himalayas that the Holocene climatic changes might have been responsible for this though, as it was stated already (Sect. 4), the range of Holocene climatic change does not seem to have been very strong.

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## **Soil Chemistry and Plant Performance – Ecological Considerations**

By Germund Tyler and Ursula Falkengren-Grerup

### **1. Introduction**

The preference of plants for particular soil conditions is a widely recognized ecological principle and was a main concern in classical plant ecology. In spite of a long research tradition, however, mechanisms involved are still far from adequately explained. Moreover, conclusions concerning wild-growing plants have often to be adopted from progress made in agrochemical and plant physiological work, usually performed on cultivars of crop plants.

Soil and soil solution properties of natural and seminatural sites usually differ greatly, sometimes in orders of magnitude, with regard to ion concentrations and proportions from those of cultivated soils or those used in plant physiological studies. It is, therefore, difficult to transfer quantitative, sometimes even qualitative, information from work with crops to gain information about mechanisms and reactions controlling the edaphic distribution of the native flora in any geographical region.

Soil acidity is a superior property which, directly or indirectly, regulates growth, general performance and field distribution of plants. Numerous conditions are controlled by, or correlated with, soil acidity, including  $H^+$  and  $Al^{3+}$  concentrations of the soil solution, base saturation, solubility of Fe, Mn and other trace elements, solubility and chemical speciation of phosphate, nitrifying capacity, etc. (Kinzel 1982; Bergmann 1988; Marschner 1991; Tyler 1993). Soil pH, or base saturation, is often the single variable which accounts for a main proportion of the statistical variability in relationships between soil chemistry and distribution of plants and macrofungi, though a simultaneous consideration of other soil factors usually increases the variability accounted for (Tyler 1976, 1996b; Hansen and Tyler 1992).

Correlative work on soil chemistry and field distribution of plants may be a suitable and often indispensable way of characterizing plant behaviour and detecting or defining problems to be treated experimentally. In order to elucidate control mechanisms, however, a variety of experimental approaches and techniques have to be applied. Plants

should be exposed to conditions which are possible to define and control but, on the other hand, do not deviate so much from conditions prevailing in the field that the ecological relevance is lost. These often opposing claims are difficult to meet and it is a real challenge of future work to bridge the gap between field ecology and plant physiology/biochemistry.

One classical problem in plant ecology is the presence of 'calcifuge' and 'acidifuge' species. 'Calcifuge' behaviour – inability to establish and develop on high pH (mainly calcareous) soils – is usually not caused by an incapability of the plant to tolerate a soil solution pH of 8.0–8.5, but rather by an inability of the plant to render certain minerals available for uptake. In particular, phosphate (Tyler 1992; Tyler and Olsson 1993), Fe and Mn (Bergmann 1988) are quite little soluble in such soils and plants have to exude solubilizing compounds to render these elements available. Certain low-molecular organic acids and their salts (Gerke et al. 1994), as well as siderophores (Römheld and Marschner 1986; Marschner and Römheld 1994), are active in this release and plant uptake of sparingly soluble nutrients from soils and great inherent differences in exudation pattern exist between 'calcifuge' and 'calcicole' species (Ström et al. 1994; Tyler and Ström 1995).

A major concern, at least over large areas of central and northern Europe, is soil acidification. The pH of most forest topsoils in southern Sweden has decreased by 0.5–1.0 pH unit, sometimes even more, since the middle of this century (Falkengren-Grerup 1987; review by Nilsson and Tyler 1995). Silicate soils have changed from a stage where base cations were the main acidity buffering agents, to another stage where solubilization of Al has adopted this role (Ulrich 1981; Ulrich and Pankrath 1983). Below a soil solution pH of ca. 4.5 any decrease in pH causes a release of toxic  $\text{Al}^{3+}$  (Bergkvist 1987). This is particularly true of soils low in organic matter, where soil solution concentrations of free (non-chelated)  $\text{Al}^{3+}$  may exceed  $0.1 \text{ mmol l}^{-1}$ , a level which causes severe damage to roots of many, may be most, vascular plants.

There is considerable field and experimental evidence for the great importance of Al toxicity to or Al interference with nutrient uptake in plants (Foy 1984; Runge 1984; Andersson 1988). However, the  $\text{H}^+$  concentration or activity may per se be even more decisive in organic soils, e.g. in the mor horizon of forests. In such soils acidity may be quite high but concentrations of free  $\text{Al}^{3+}$  low, because of a small pool of total Al and the presence of less toxic Al complexes with humic constituents (Falkengren-Grerup and Tyler 1993a; Gerke 1994). It is also highly probable that the differing ability of plants to tolerate soil acidity is partly controlled by differing exudation properties, variously ameliorating the environmental conditions of the rhizoplane. Aluminium chelates or complexes with organic ligands of low molecular weight are usually also less toxic than free  $\text{Al}^{3+}$  (Lee and Foy 1986; Suhayda and Haug 1986).

Nitrogen in soil plays an often decisive role in the primary productivity of a variety of ecosystems. The reversion of N as a factor limiting tree growth is sometimes considered to cause nutritional disorders which might violate the economy of forestry. N 'saturation' is a condition where the supply of available N exceeds the accumulation capacity of plants and soil microorganisms. It may be a consequence of excessive atmospheric deposition and increase cation losses by nitrate leaching, thereby accelerating soil acidification and Al solubilization. The distributions of numerous plant species are considered to be influenced by the availability of N, though it is often difficult to distinguish effects of N from the influence of other soil chemical properties, e.g. phosphate status or soil acidity, without conclusive experimental data. There is also evidence for the importance of N form to the performance of many plants. Preference for nitrate over ammonium as the N source may also ameliorate soil acidity, whereas preference for ammonium acts in the opposite direction.

The aims of this chapter are to highlight some recent progress in research on soil – plant interactions with a focus on non-cultivated plants and ecological considerations. Particular attention will be paid to possible mechanisms controlling plant growth on highly acidic and on calcareous soils, and to plant – N relationships.

## 2. Acidifuge Behaviour and Toxicity of Acid Soils

Increasing soil acidity exerts a profound influence on many soil chemical properties of importance to the performance of plants. In northern-central Europe, ion exchange with  $H^+$  has reduced the plant-available pools of base cations, as input by weathering and mineralization has usually been unable to compensate for losses due to leaching and increased storage in plant biomass. In many soils, base cation pools have decreased to such an extent that Al has taken over as a main acidity buffering mechanism.

Many plants are unable to tolerate those chemical conditions which now prevail in many sites, where the plants have been established since long. In southern Swedish forests, where the acidification is particularly pronounced, species like *Galium odoratum*, *Pulmonaria obscura*, *Mercurialis perennis* and *Polygonatum multiflorum* are nowadays less frequent than in earlier decades (Falkengren-Grerup 1986, 1995a). Other deciduous forest plants, e.g. *Hepatica nobilis*, seem to have lost their power of rejuvenation from seeds, as no seedlings or establishment of new plants seem to occur, except quite locally in the least acid sites.

Adverse conditions in acid soils are usually related to high concentrations of soluble  $H^+$  or  $Al^{3+}$ . Locally or accidentally, soluble Mn might rise to phytotoxic levels, though Mn toxicity is difficult to verify experi-

mentally at realistic soil solution concentrations. In an experiment with the 'acidifuge' *Melica ciliata*, grown from seeds originating from a limestone population,  $0.5 \text{ mmol l}^{-1}$  of  $\text{Mn}^{2+}$  did not influence growth (Tyler 1993). However, Mahmoud and Grime (1977) demonstrated a susceptibility to Mn in native grasses in the  $0.1\text{--}4 \text{ mmol l}^{-1}$  range which was inversely related to the ability of these plants to colonize acidic soils. Concentrations exceeding ca.  $0.5 \text{ mmol l}^{-1}$  in nutrient solution reduced root elongation in *Geum urbanum* (Waldren et al. 1987). Concentrations of Mn in acid soil solutions do, however, rarely exceed  $0.2 \text{ mmol l}^{-1}$ . Typical ranges of  $0.01\text{--}0.05 \text{ mmol l}^{-1}$ , occasionally  $0.2 \text{ mmol l}^{-1}$  were measured in the top horizons of dystric cambisols in beech forest of southern Sweden, using high-speed centrifugation technique to expel soil solutions (Falkengren-Grerup and Tyler 1993c).

Physiological mechanisms accounting for Al ion toxicity in plants include inhibition of root cell division and/or elongation, the latter process also pertinent at moderate Al ion concentrations. Al ions may act on the cytoplasm, as well as on plasma membrane structure and function—callose formation sometimes reminding of a reaction to wounding may occur in the rhizoderm and in the cells of the root cap (Wissemeier et al. 1987), interfering with activities indispensable to root growth and development, as reviewed by Marschner (1991). Even when Al ion activity does not cause any mechanical damage to the cells, antagonistic reactions with essential ions, including phosphate and Ca, may evoke nutrient deficiency but also ameliorate the toxic effects of Al (Adams and Hathcock 1984; Rengel 1992; Brunet 1994). Ameliorative effects of Ca are, however, debated and sometimes not possible to demonstrate (e.g. Falkengren-Grerup et al. 1995b; Løkke et al. 1996).

A high acidity of the soil solution increases the demand on the plant cell to maintain a functionable pH. If the capacity of this  $\text{H}^+$  pump is reduced or inferior,  $\text{H}^+$  damage to membranes and other cell constituents is likely to occur, unfavourably changing the permeability conditions of the membranes and ultimately inhibiting plant growth. Under ecologically relevant conditions, it is usually difficult to discriminate between adverse effects of elevated  $\text{H}^+$  or Al ion concentrations, because these two variables tend to be closely correlated at moderate to high soil acidity. However, in strongly acid organic topsoils, e.g. forest mor horizons, where the amount of total Al is rather low and soil solution Al is mainly complexed to soluble organic ligands, a high  $\text{H}^+$  activity may determine the plant limits of existence, as shown experimentally with several forest species (Falkengren-Grerup and Tyler 1993a). Andersson (1992), using solutions closely similar to those of acid soils, exposed *Galium odoratum* in a flowing solution system without recirculation to maintain the chemical matrix. Even when Al was excluded, growth rate was reduced to about half at pH 4.5, compared with pH 5.0, and growth was completely inhibited at pH 4.0.



Also, nutrient deficiency in highly acid soils may for similar reasons be difficult to discriminate from Al ion or  $H^+$  toxicity. It has been demonstrated that amendment of strongly acid forest soils with a variety of plant nutrients (including base cations, N and P), without raising soil pH, failed to improve growing conditions for a variety of forest plants (Falkengren-Grerup and Tyler 1992; 1993b; Falkengren-Grerup 1995c). However, raising the soil pH by adding  $CaCO_3$ ,  $CaCO_3 + MgCO_3$ , or even only  $SrCO_3$  to a strongly acid dystic cambisol resulted in a consistent and considerable improvement in growth and performance of the plants. The fact that  $SrCO_3$  alone was nearly as efficient in this respect as the 'nutrient' carbonates indicates that these soils were primarily not deficient in Ca or Mg, in spite of low base saturation values.

Numerous tests have been performed on effects of Al on growth, nutrient uptake and general performance of plants. Results reported on toxicity limits seem to differ greatly, according to species, growing conditions, solution matrix used, etc. Most studies reported so far were conducted in nutrient solutions which greatly deviated from the chemical composition of acid soil solutions, usually being much higher in most of the essential elements. Divalent base cations, in particular  $Ca^{2+}$ , but also phosphate, are able to decrease or modify the Al toxicity of nutrient solutions (Alva et al. 1986; Rengel 1992; Keltjens and Tan 1993). However, in a field study on soil factors related to the distribution of plants in acid deciduous forests (Falkengren-Grerup et al. 1995b), both the exchangeable and the soil solution Ca:Al ratios proved generally inferior to Ca or Al alone in accounting for the distributions. Any evidence for Ca – Al interactions seems difficult to generalize from solution culture experiments to conditions prevailing in the field.

The speciation of Al in the solution is of great importance to toxicity limits and reactions. Al ions are easily complexed or chelated to organic ligands, a reaction which usually reduces toxicity (Taylor 1988). Humic ('fulvic') acids, which are present in highly differing concentrations in most soil solutions may decrease Al toxicity. A further circumstance, which may violate predictions of Al toxicity of soils, is that plants may have greatly differing capacities to modify their rhizosphere environment. Plant roots, as well as germinating seeds, exude a variety of organic compounds, including chelators of, e.g., Al ions into the soil solution. The ecological importance of this mechanism to the Al toxicity of soils is still little known. However, it has been demonstrated with some cultivated plants that differences in Al sensitivity, e.g. between cultivars, are related to differences in exudation rates and patterns (Suhayda and Haug 1986; Horst et al. 1990; Miyasaka et al. 1991). Complexation to organic acid ligands might be a principal mechanism which reduces the phytotoxicity of cationic Al in soil and nutrient solutions (Hue et al. 1986; Taylor 1988).

A further problem in evaluating possible Al toxicity of soils is the circumstance that also polynuclear Al species may be found, or have been calculated to occur, in soil or nutrient solutions. According to Kinraide (1991) a highly toxic cationic  $Al_{13}$  species might form in solutions at least under conditions of pH instability. It has been postulated (Kinraide 1990) that a polymeric Al species may form from aluminate,  $Al(OH)_4^-$ , also in moderately alkaline media. Aluminate occurs at low concentrations in calcareous soil solutions (Fuller and Richardson 1996; Tyler 1994a). The extent to which polynuclear cationic Al species do have an ecological importance under field conditions is, however, essentially unknown and further studies are needed to elucidate this problem.

In closed or recirculating solution systems, if not subject to frequent replacement or regeneration, exudates or metabolites may accumulate, causing changes in Al speciation and toxicity. Though not being entirely without objection, systems using flowing solution culture without recirculation allow a comparatively much better control of the chemical speciation of elements supplied than is possible with most other techniques. Using such systems Andersson and Brunet (1993) reported growth retardation of the forest grass *Bromus benekenii* from  $10 \mu\text{mol l}^{-1}$  of  $Al^{3+}$  in a solution (pH 4.2) composed to closely mimic an average for acid forest soil, but lacking organic constituents. An  $Al^{3+}$  concentration of  $20 \mu\text{mol l}^{-1}$  (Ca:Al molar ratio 12.5; pH 4.3) in a similar system caused severe reduction in root growth of young plants of *Allium ursinum* (Andersson 1993).

Soil solution concentrations of monomeric, 'quickly' reacting  $Al^{3+}$ , analysed according to the method of Clarke et al. (1992), may often be much higher than  $10\text{--}20 \mu\text{mol l}^{-1}$ . Concentrations as high as  $80 \mu\text{mol l}^{-1}$  were measured in soil solutions obtained by high-speed centrifugation of samples at field moisture from the humus layer of dystric cambisols (Andersson and Brunet 1993). Even higher concentrations have occasionally been measured with a similar technique in highly acidic mineral topsoils (Tyler 1993). In such sites only few vascular plant species are able to establish and develop successfully, e.g. *Deschampsia flexuosa*, *Carex pilulifera* and *Rumex acetosella*.

One mechanism of Al tolerance rarely considered is the possibly differing ability of plants to recover from damage caused by periods of adverse soil conditions. The concentration of toxic Al species in the solution of a particular soil is probably far from constant. It may be subject to seasonal or episodic variability as a result of fluctuations in acid input or production (nitrification, cation uptake, etc.) or soil moisture. Drought during 2 summer months almost doubled the  $H^+$  concentration in the topsoil of beech forests and, thereby, influenced soil chemistry accordingly (Falkengren-Grerup 1994). Though energy costs for root reestablishment could be more than marginal and negative in the interspecies competition, those plants which are able to replace damaged

roots rapidly should have an advantage. In a study of forest plants from slightly to moderately acid soils (Quist 1995) *Galium odoratum* was irreversibly damaged by 2-week episodes of  $20 \mu\text{mol l}^{-1}$  of  $\text{Al}^{3+}$  or by a low pH (4.0). It was unable to recover when transferred to more suitable conditions. *Lamium galeobdolon* was about equally sensitive to Al episodes, but it recovered much better. *Galium odoratum* has become less frequent in recent decades, at least in southern Sweden, when forest soils have acidified and Al solubility increased, whereas the frequency of *Lamium galeobdolon* has not been reduced. Differences in the capacity to regenerate seem to be the probable explanation.

Large-scale field evidence for the importance of Al as a soil factor influencing plant distributions is given in a recent survey on soil – plant species relationships covering ca. 650 sites in southern Scandinavia (Tyler 1997). From a maximum in neutral or slightly alkaline (calcareous) sites, species richness tends to decline regularly but rather slowly with increasing soil acidity down to a soil solution pH of ca. 5 (pH-KCl 4.5). At this point there is a sharp 'bend' of the regression line for pH on species richness to a considerably greater inclination with increasing acidity, perfectly coinciding with the point where  $\text{Al}^{3+}$  starts to be released from the solid phase into the soil solution (Fig. 1).

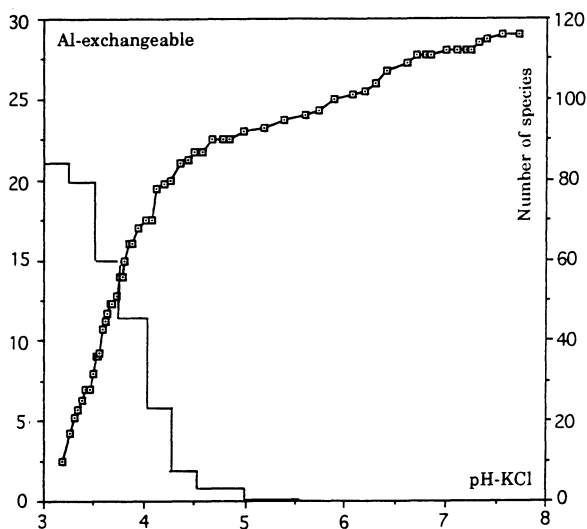


Fig. 1. Changes in species richness and exchangeable soil  $\text{Al}^{3+}$  ( $\mu\text{mol g}^{-1}$  dry weight) along a soil pH gradient. Graph is based on species composition and soil data from ca. 650 rock meadow sites in southern-south-central Sweden, calculated in groups of 10 according to similarly in soil pH. (Tyler 1997)

### 3. Calcifuge Behaviour and Capacity of Plants to Modify Their Soil Environment

It is well known that numerous vascular plants of the European flora never, or only occasionally, occur on calcareous soils, or other soils with a high pH, in spite of the fact that species richness is generally higher on such soils. Simple experiments are usually sufficient to demonstrate the low or lacking ability of such 'calcifuge' plants to develop and grow on carbonate, e.g. limestone, soils. It might also be concluded, from experiments with nutrient applications, that a deficiency of minerals or inability of calcifuge plants to solubilize or metabolize less readily available elements in such soils are major conditions and processes involved.

The chemistry of calcareous soils differs greatly, not just with respect to pH or Ca concentrations, from the chemistry of acid soils. The acidity is kept low by the buffering influence of  $\text{CaCO}_3$ , the typical soil solution pH ranging from 8.0 to 8.5. Moreover, the soil solution is often oversaturated with  $\text{HCO}_3^-$  owing to the production of  $\text{CO}_2$  from the respiration of organisms. On the contrary, there is almost no easily exchangeable or soil solution Fe and Mn ions in aerated limestone soils, as these elements are present there in less soluble forms (Table 1). Exchangeable or soil solution phosphate is also consistently quite low in natural or seminatural limestone soils. Apart from being organically bound, limestone soil phosphate is contained in insoluble apatite-like minerals. The small instantaneous amounts of phosphate in limestone soil solutions are present as the  $\text{HPO}_4^{2-}$  species which, moreover, may be less easily absorbed by plant roots than the  $\text{H}_2\text{PO}_4^-$  species prevailing in acid soils.

**Table 1.** Inorganic chemical composition of *soil solution* from root horizon of limestone soils and acid silicate soils. Ionic concentrations calculated as  $\mu\text{mol l}^{-1}$ . Soil solutions were expelled by high-speed centrifugation technique using freshly sampled soil at field moisture. Figures shown represent normal ranges in non-forested natural – seminatural ecosystems in southern Sweden

	Limestone soil	Acid silicate soil
pH	7.5–8.5	3.5–4.5
$\text{Ca}^{2+}$	2000–3000	200–1000
$\text{Fe}^{2+}/\text{Fe}^{3+}$	< 1	2–20
$\text{Mn}^{2+}$	< 0.5	2–20
$\text{Al}^{3+}$	< 0.5	10–200
$\text{Al}(\text{OH})_4^-$	2–10	< 0.5
$\text{HCO}_3^-$	1000–3000	< 1
$\text{HPO}_4^{2-}$	< 1–5	< 0.5
$\text{H}_2\text{PO}_4^-$	< 0.5	5–50

The knowledge about mechanisms controlling 'calcifuge' behaviour of plants is, however, far from conclusive. Inability to solubilize or absorb Fe has attracted most attention in agricultural research and numerous studies on the Fe nutrition of crops are available, as reviewed by, e.g., Bergmann (1988), Bienfait (1989) and Abadía (1995). 'Lime chlorosis', usually considered to reflect deficient Fe status, has also been reported from plants of non-cultivated soils (Grime and Hutchinson 1967; Grime and Hodgson 1969; review by Kinzel 1982). One reason for the focus on Fe in agrochemical research is that Fe deficiency is difficult to correct by traditional fertilization methods, because Fe added to calcareous soils is rapidly immobilized there.

However, difficulties to render phosphate available seem to constitute primary limitations to establishment and growth of most wild calcifuge species tested so far. Seedling establishment in, e.g., *Viscaria vulgaris*, *Rumex acetosella* and *Silene rupestris* was not successful in limestone soils of Archean and Ordovician origin unless soluble  $\text{CaHPO}_4$  was supplied (Tyler 1992; Tyler and Olsson 1993). Growth of adult plants in limestone soil, e.g. *Carex pilulifera*, *Deschampsia flexuosa*, *Holcus mollis*, *Luzula pilosa*, *L. campestris*, *Scleranthus perennis*, *Nardus stricta*, *Potentilla erecta*, *Rumex acetosella*, *Veronica officinalis* and *Agrostis capillaris*, was highly favoured by  $\text{CaHPO}_4$  addition, being a prerequisite for growth in some of the species. Poor growth of calcifuges in limestone soils was related to low tissue concentrations of phosphorus (Tyler 1994b, 1996b).

Particularly when supplied with adequate amounts of phosphate for normal biomass production rates, several calcifuges tend to develop foliar chlorosis when grown on limestone soils. This indicates that they are facing new nutritional problems when the primary growth limitations by phosphate have been reversed. In *Carex pilulifera* and *Galium saxatile* chlorosis uniformly comprises the entire leaf lamina, whereas intercostal chlorosis is more typical to *Veronica officinalis*. The chlorotic symptoms resemble those developed from Fe deficiency in crops (Bergmann 1988). Repeated spraying with Fe(III) citrate may facilitate the development of non-chlorotic leaves, whereas leaves already produced usually stay chlorotic, probably because of a low mobility of Fe in the plant.

Different mechanisms seem to be responsible for Fe deficiency in calcareous-soil-grown plants. Many calcifuge species, e.g. *Carex pilulifera*, reduce their uptake of Fe in above-ground biomass to such an extent, when forced to grow on calcareous soil, that total tissue concentrations become low in chlorotic leaves. However, Fe chlorosis is not always accompanied by a lower total tissue concentration of Fe (Mengel and Scherer 1984) and plant uptake of Fe is not consistently lower from calcareous than from acid soils. It may sometimes even be higher, as shown, e.g., for *Chamaenerion angustifolium* (De Neeling and Ernst

1986). *Veronica officinalis* is able to retain or even increase its uptake of Fe, when grown in calcareous soil, but still it develops chlorosis. Current research has shown that in *Carex pilulifera* much Fe is 'trapped' already on the surface of the roots and, as a consequence, less Fe enters the plant tissues. In *Veronica officinalis*, less Fe is immobilized in this way and more Fe is transported to the leaves. Instead, a much smaller proportion of the element than is the case in acid-soil-grown plants is retained in the leaves in forms which are extractable with Fe chelating agents, e.g. phenanthroline (Zohlen and Tyler 1997). It is, therefore, likely that much of the tissue Fe in calcareous-soil-grown *Veronica officinalis* is immobilized in forms which are metabolically less active in the plant.

The knowledge about mechanisms responsible for tissue immobilization of Fe are not fully conclusive. Bicarbonate ions in soil solutions and plant biomass seem to be involved (Mengel et al. 1984). A high  $\text{HCO}_3^-/\text{OH}^-$  concentration around the plasma membrane of the root cells probably inhibits the Fe(III) reductase activity, decreasing the transport rate of Fe to the shoots. A similar mechanism might be responsible for an immobilization of Fe actually transported to the leaves. The apoplastic pH of chlorotic leaves may be high and acid treatment may sometimes reverse the chlorotic symptoms (Mengel 1994).

Differences among species in their response to environmental stress, e.g., displayed as acidifuge – calcifuge behaviour, may to a considerable extent be due to differing ability of plants to modify their soil environment. Differences among plants in their production and excretion of mineral-nutrient solubilizing compounds, or differences in the proportion of cation-to-anion uptake by their roots, are mechanisms involved. The main way of plants to modify the latter proportion is by preferential uptake of either ammonium or nitrate as the N source, to be discussed in Section 4.

Excretion (exudation) of organic compounds may occur from most kinds of plant tissue, though primarily from limited areas on fine roots. A variety of compounds are exuded, including sugars and polysaccharides, amino, phenolic and low-molecular-weight carboxylic acids, and extracellularly active enzymes. In grasses, metal chelating compounds, commonly called phytosiderophores, are important (Marschner and Römheld 1994). Exuded compounds might also be reabsorbed, either unchanged or changed by, e.g., chelation. Many compounds, including several phenolic acids and their derivatives, may have allelopathic effects of importance on the intra- and interspecific competition of plants (Abdul-Rahman and Habib 1989; Yamane et al. 1992). Others, e.g. sugars and amino acids, play an important role in the establishment of a rhizosphere microflora or in the development of mycorrhiza. These considerations are, however, beyond the scope of this chapter.

Highly essential in accounting for calcicole – calcifuge behaviour are mainly the phytosiderophores and the low-molecular-weight carboxylic

acids, owing their capacity of releasing critical mineral nutrients from soils. This capacity is particularly important in calcareous soils. The phytosiderophores are currently only known to be produced within the grass family, *Poaceae*. They have a specific capacity to facilitate plant uptake of Fe and other cationic micronutrients (Marschner and Kissel 1986; Jolley and Brown 1989; Römheld 1991). Exudation rates may be enhanced by deficiency of such nutrients, especially Fe, which may be particularly pronounced in calcareous soils. Several calcifuge wild grasses, grown in hydroponic culture devoid of Fe, were able to exude about one order of magnitude more Fe-mobilizing compounds than similarly treated calcifuge grasses (Gries and Runge 1992, 1995). The nature of the mobilizing compounds was not determined in these studies but assumed to be mainly phytosiderophores. Siderophore-like compounds are also produced by soil bacteria and it might be postulated that plants are able to utilize also these substances in their micronutrient retrieval. However, two bacterial siderophores tested by Bar-Ness et al. (1992) were not efficient as Fe sources in cereals, even if supplied by stem injection, and the possible participation of these compounds in the acquisition of nutrients by plants deserves future attention.

More generally valid in plants as a mechanism for mineral nutrient release is the exudation of low-molecular-weight organic (carboxylic) acids (LOAs). Among these, the monocarboxyls formic, acetic and lactic acid, the dicarboxyls malic, succinic and oxalic acid, and the tricarboxyls citric, isocitric and aconitic acid are most often found (Vancura 1964; Kovacs 1971; Ström et al. 1994). Also, puruvic and tartaric acid and, more occasionally, a variety of other organic acids are sometimes encountered in plant root exudates. Proportions of acids exuded are to some extent species-specific (Vancura and Hovadik 1965), but major general differences in this respect exist between plants of contrasting soils.

These comparatively weak acids are variously efficient in solubilizing, e.g., Fe and phosphate from soils. Citric acid is a powerful solubilizer of Fe, as it forms water-soluble complexes, the Fe of which is at least partly available for plant uptake. Oxalic acid/oxalate is particularly efficient in releasing phosphate from limestone soils by forming an insoluble complex with Ca, thereby mobilizing phosphate for uptake. Other LOAs tested, especially the monocarboxylic acids, are much less efficient in these respects (Tyler and Ström 1995; Ström 1997).

It was recently demonstrated that calcicole species, representing many taxonomic groups, are characterized by a much higher exudation rate of two- and three-carboxylic acids than acidifuge species when exposed to nutrient-poor conditions. Calcicole plants, as an average, exuded three- to ten-fold more citric and oxalic acid per unit of tissue and root weight than calcifuge plants (Ström et al. 1994; Tyler and Ström 1995). The difference proved valid for adult individuals as well as seed-

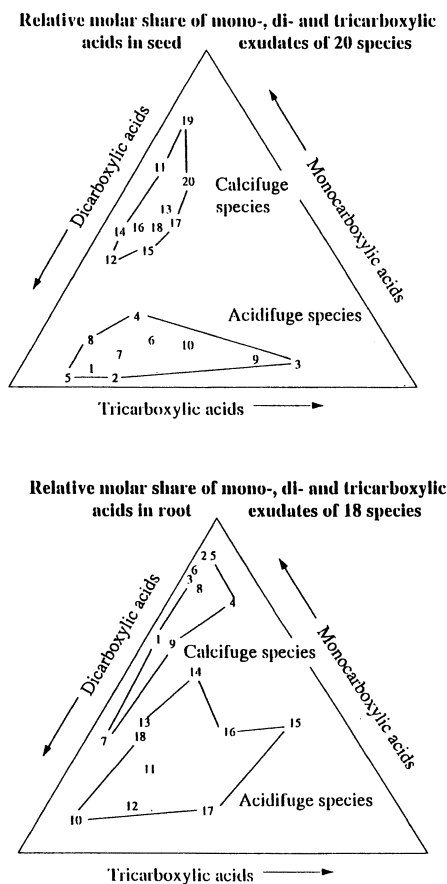


Fig. 2. Proportions of mono-, di- and tricarboxylic acids in exudates from germinating seeds (*above*) and roots of adult plants (*below*), exposed to nutrient-poor solutions. Each study is based on 9–10 'acidifuge' and 9–10 'calcifuge' species of herbaceous and gramineous plants. *Numbers* in the graphs represent means of different species. (Redrawn from Tyler and Ström 1995, *upper graph*; and Ström et al. 1994, *lower graph*)

lings and germinating seeds (Fig. 2). There was a general difference also for other di- and tricarboxyls, whereas concentrations of monocarboxyls measured were similar or less consistent with the edaphic origin of the species. This would mean that the capacity of calcifuge herbaceous plants and sedges to render sparingly soluble elements available for uptake from calcareous soils is really limited, as no other system is known with a functionally similar efficiency in those plants. Moreover, judging from the few species of grasses studied so far, these exudation differences between contrasting edaphic groups seem to be valid also for species of the *Poaceae*.

In a recent study on rhizosphere soil solution chemistry under two calcifuges (*Deschampsia flexuosa* and *Viscaria vulgaris*) and two calcicoles (*Gypsophila fastigiata* and *Sanguisorba minor*), all cultivated in an



'intermediate' soil, the differences between contrasting edaphic species groups were further confirmed (Ström 1997). The concentrations of di- and trivalent carboxylic acids were much higher in the soil solutions of the two calcicoles, whereas there was no difference in the monocarboxylic acid concentrations.

However, it is currently not possible to obtain real quantitative data on the *in situ* exudation rates in soils, because acids are immobilized or decomposed to a variable degree. Di- and tricarboxylic acids added at  $10 \mu\text{mol l}^{-1}$  to samples of the topsoil horizon of dystric cambisols were almost instantaneously immobilized to a high degree (oxalic acid to 83–87%, citric acid to 75–87%), whereas the monocarboxylic acetic and lactic acid were almost quantitatively recovered in the solutions after exposure to the soils (Shen et al. 1996). Microbial decomposition was not a main explanation of these findings, but, rather, unequal chemical precipitation of sorption properties among acids. This is consistent with their differing ability to interact with soil mineral nutrients, as discussed above. Immobilization of, e.g., oxalic acid is supposed to be due to precipitation of complexation with cations like Ca and Al, thereby liberating phosphate for plant uptake. The higher the soil pH, the higher is the degree of dissociation of the acid and the higher its activity in interacting with the solubility of soil minerals.

It may be argued whether the concentrations of LOAs exuded by plant roots are high enough to exert any profound influence on mineral solubility. Concentrations of major LOAs in bulk topsoil solutions are usually in the  $1\text{--}25 \mu\text{mol l}^{-1}$  range. However, close to the actively exuding areas, concentrations should be several orders of magnitude higher, attaining concentrations experimentally found to extract large amounts of phosphate, Fe and Mn from soils. As growing fine roots are continuously exposing 'fresh' soil to the action of exudates, mineral nutrients solubilized in this way should be a main source to plants capable of a quantitatively and qualitatively adequate exudation.

#### 4. The Importance of Nitrogen Availability

Nitrogen is a limiting nutrient in a wide range of ecosystems. Addition of N to forests, grasslands and mires usually increases biomass production. Species composition may change and the richness decrease as late successional species which are superior N competitors displace early successional species, and long-lived species with a high stature are favoured (Tilman 1987; Tilman and Wedin 1991).

The increased N deposition in large parts of Europe has changed the natural vegetation as shown in a comprehensive review made to set critical loads for negative effects of N on vegetation (Bobbink et al. 1996). It is, however, difficult to assess the causal relationships in an

environment that alters in several respects, e.g. through soil acidification, land use and management. It is also impossible to state at which deposition levels the changes occurred, as such long-term monitoring studies are scarce or non-existent.

Field studies often show an increase in N-demanding species in areas with a significant amount of deposition (Falkengren-Grerup 1995a), while such effects are less obvious where the deposition is lower or the observation time short (Brunet et al. 1996; Falkengren-Grerup and Brunet 1996). Fertilization experiments have the disadvantage that the historical and current deposition cannot be separated from the treatment effects, a fact that is often overlooked in the interpretation of experiments, and that responses to low doses require long observation periods. This is exemplified by a study from a spruce forest in the middle of Sweden, where some herbs showed a weak response to  $5\text{--}40\text{ kg N ha}^{-1}\text{ year}^{-1}$  after 1 year of treatment, gradually becoming stronger during the 5-year experiment (Kellner and Redbo-Torstensson 1995). If the soils are N-saturated, a further addition will rather answer the question how the vegetation responds to soil acidification (Falkengren-Grerup 1993).

Several countries are taking part in a European project with N added or removed from ambient atmospheric deposition to simulate changes in N deposition in coniferous forests. The deposition ranged between 13 and  $59\text{ kg N ha}^{-1}\text{ year}^{-1}$  (Gundersen et al. 1997). The needle N concentration was positively and, generally, the K and Mg negatively related to the deposition (Boxman et al. 1997b). Tree growth increased when N was removed (Boxman et al. 1997b) and there was a tendency for stabilization and a gradual recovery of fine-root growth (Persson et al. 1997). As the ground-layer was poorly developed in most sites, only a few observations could be made. In one site *Rubus* spp. and *Dryopteris dilatata* decreased rapidly after N removal and no new colonization was found during the 6-year experiment (Boxman et al. 1997a).

There are other ways of examining the N-related processes in nature. An increased availability, or a surplus of N relative to other nutrients, can cause higher tissue concentrations of N and warn against imbalances in the plant and, in the long run, in the ecosystem. Several studies show that plants accumulate amino acids in response to experimental N additions and that this is related to the variability in N deposition found in transects over Europe or within a country. The specific amino acids vary according to both plant species and the level of N accumulation. The amino acids with the most pronounced increase were arginine in coniferous trees (Edfast et al. 1990) and asparagine in *Fagus sylvatica* (Balsberg-Påhlsson 1992). The arginine content also decreased rapidly when N was removed in a roof experiment in the Netherlands (Boxman et al. 1997a). For *Vaccinium* spp., *Deschampsia flexuosa* and *Epilobium angustifolium* the amino acids which reacted on increased N supply varied considerably (Näsholm et al. 1994). High amounts of total organic

N and nitrate were also positively related to nitrate richness in the soil for 48 plant species of natural habitats (Gebauer et al. 1988).

The natural abundance of  $^{15}\text{N}$  in plants is determined by the isotope ratio of the different N forms taken up by the plant, the discrimination in uptake and leaching of  $^{15}\text{N}$  and  $^{14}\text{N}$ . Analyses of  $^{15}\text{N}$  were made in a *Larix - Picea - Fagus* stand to study the N sources utilized by trees, shrubs and grasses (Gebauer and Dietrich 1993). All trees used similar N sources,  $^{15}\text{N}$  being slightly negative, in spite of having different rooting depths, and they might have used the soil organic layers preferentially for their N uptake. Grasses and non-ericaceous shrubs did not deviate much from the trees, while fungi had a positive  $^{15}\text{N}$ , i.e. a large uptake from the organic humus layer. The ericaceous shrubs were most depleted in  $^{15}\text{N}$ . Analysis of  $^{15}\text{N}$  can improve the understanding of different strategies of plant N uptake in an ecosystem (Michelsen et al. 1996).

Species with a low but stable N uptake are considered to be more vulnerable to an increased N supply than species with a higher plasticity (Grime 1979; Tilman and Wedin 1991). Nitrogen-fixing species are more competitive in soils low in N, but they can also take up a substantial amount of  $\text{NO}_3$  and  $\text{NH}_4$  released in the soil. Species in fertile habitats generally have a higher plasticity and respond with a higher biomass to an increased availability of a limiting nutrient (Grime et al. 1986; Boot and Mensink 1990; van de Vijver et al. 1993). Apart from  $\text{NH}_4$  and  $\text{NO}_3$ , plants may be able to take up considerable amounts of organic N, either directly or transported by mycorrhizal fungi (Read et al. 1989; Chapin et al. 1993). Most research has been devoted to ectomycorrhiza, while arbuscular fungi are symbionts with grasses and herbs and probably have different abilities or strategies (George et al. 1995; Michelsen et al. 1996). Arbuscular fungi are able to transport considerable amounts of inorganic N to their hosts (Johansen et al. 1993a,b).

Plant-available inorganic N is often estimated as net mineralization under laboratory or field conditions over several weeks (potential mineralization). Conditions set in the laboratory are naturally of importance as, for example, temperature and moisture are positively related to the mineralization rate (Fisher and Whitford 1995; Stenger et al. 1995). Disturbed soil columns may give twice as high values as undisturbed columns (Stenger et al. 1995), but may also cause problematic changes in the N dynamics (Hook and Burke 1995). The seasonal variation is large and even soil sampling at a monthly interval may give rates deviating by 50% (Dendooven et al. 1995). Other methods of estimating plant-available N, which have proven to be well correlated with the N uptake by a particular species, are chemical extractions with varying soil moisture, microbe treatments and incubations (Serna et al. 1992; Stockdale and Rees 1994). These methods are applicable when relative N amounts over space or time are the aims of the study.

The inorganic N produced in a soil is dependent on the amount and quality of the organic matter, the soil structure and chemistry and the microbes active in ammonification, nitrification and denitrification. The availability of N for perennial ryegrass could to 90% of the variance be accounted for by soil inorganic N, mineralized N in an incubation experiment and total N in the macro-organic material (Warren and Whitehead 1996). The degree of nitrification is usually positively related to soil pH, while total N mineralization is often independent of pH (Falkengren-Grerup et al. 1995a).

The increased N deposition in many parts of the Western world adds substantial amounts to the soil pool and may cause leaching of  $\text{NO}_3^-$ , and thereby acidification, in nutrient-unbalanced soils. The N pools in natural soils are highest in the topsoil. Acid but productive forest soils in southern Scandinavia had 20% of the total N in the organic layers and another 30% in the uppermost 10 cm of the mineral soil (Persson and Wirén 1995). Even greater differences in the mineralization rate were found where the organic layer had a seven times higher rate, and the 0–10 cm mineral soil a double rate, as compared with the deeper soil horizons. Responses to N manipulation in the European project, including addition and removal of N to coniferous forests, showed that the lag-time before significant responses were observed varied among the processes and pools measured (Gundersen et al. 1997). The responses were generally fast in the pools of dissolved and adsorbed inorganic N in the soil, intermediate in needles and needle litter and slow in the forest floor. The pools in these sites varied between 2 and 3000 kg N ha<sup>-1</sup>.

The knowledge of plant response to the amount and form of inorganic N is substantial for agricultural soils. The results cannot, however, be easily generalized to wild plants, as these often grow at lower nutrient levels and higher soil acidity. A positive effect of N addition on many plants is found in indoor experiments. A wide range of N concentrations give increased growth, even at amounts which are much higher than found in the field. Controlled experiments are used to seek generalizations to species' response to N availability. Ten annual plants exposed to a solution containing 0.1–24 mmol l<sup>-1</sup> of  $\text{NH}_4\text{NO}_3$  increased growth up to 1.5 mmol l<sup>-1</sup>; above this level only the more nitrophilic species increased their growth (Fichtner and Schulze 1992). The shoot:root ratio generally increases with N addition and the plasticity in root characteristics are species-dependent (Robinson and Rorison 1988).

Most studies have been performed under non-limiting conditions of nutrients other than N. In recent decades, however, soil acidification in Europe has decreased the availability of many nutrients concurrent to increased N deposition. In a study on 31 forest species, the graminoids had a higher growth rate than the herbs in a low-nutrient solution with 50, 250 and 1250  $\mu\text{mol N l}^{-1}$  and they also reacted positively to increased N concentration, which was rarely found for the herbs (Falkengren-

Grerup unpubl.). The grasses seemed to have a lower demand for other nutrients than N and would thus be more competitive than herbs in acidified and N-enriched soils.

Ammonium at high concentrations in the plant is toxic and the plant therefore has to either avoid excess uptake or transform it into amino acids. Studies on effects of high  $\text{NH}_4$  concentrations on wild plants are few. A range of 0.5 to 50  $\text{mmol l}^{-1}$  in an experiment with *Picea abies* gave a peak tissue-N concentration at 5  $\text{mmol l}^{-1}$  in the solution, indicating a metabolic control of the tissue concentration (Aarnes et al. 1995).

Several experiments on the preference of wild plants for  $\text{NH}_4$  or  $\text{NO}_3$  during the 1960s and 1970s often demonstrated maximum growth with a mixture of the two N forms, but sometimes a preference for  $\text{NH}_4$  was observed in acid-tolerant species and for  $\text{NO}_3$  in less tolerant ones. High solution concentrations as compared with today's field conditions were used (cf. Falkengren-Grerup 1995b). In studies using a constant pH and field-realistic N concentrations (flowing solution culture) only one out of seven species grew better when N was supplied as  $\text{NO}_3$  alone and no species preferred  $\text{NH}_4$  (Falkengren-Grerup and Lakkenborg Kristensen 1994). In another study on 23 field-layer species, using  $\text{NH}_4$  or  $\text{NH}_4 + \text{NO}_3$ , none of the species attained a higher growth rate with  $\text{NH}_4$  (Falkengren-Grerup 1995b). The preference for the mixture  $\text{NH}_4 + \text{NO}_3$  was positively related to the soil pH of the species' field distribution.

The uptake of N as a cation or anion may influence pH of the rhizosphere. One proton is invariably released for one  $\text{NH}_4$ , while OH or  $\text{HCO}_3$  ions are usually released when  $\text{NO}_3$  is taken up. pH changes may, however, also fail to appear (Marschner and Römhild 1983). Whether the pH changes occur depend on the plant species, its nutritional status and the pH buffering capacity of the soils (Marschner 1991). Moreover, increased uptake of  $\text{NO}_3$  may accelerate the uptake of cations, e.g. K, thereby reducing any net influence on pH. All  $\text{NO}_3$  taken up has to be reduced and assimilated in amino acids and the energy cost of  $\text{NO}_3$  uptake is therefore considerably higher than of  $\text{NH}_4$  (Gutschick 1981).

The knowledge of N allocation in the soil is of great importance as allocation constitutes the basis for competition for  $\text{NH}_4$  among heterotrophs, nitrifiers and roots (Riha et al. 1986). Plants may compete with nitrifiers for  $\text{NH}_4$ , making an uptake of both N forms potential also in nitrifying soils (Jackson et al. 1989; Davidson et al. 1990; Verhagen et al. 1994). The decomposers, on the other hand, allocate for their own growth the amount that maximizes their population biomass, leaving the remainder available for the plants (Harte and Kinzig 1993).

The N indicator values of Ellenberg (1992) characterize species occurrence in central Europe in relation to the N pools available during the vegetation period. The applicability of the indicator values is discussed in the literature and they are often found to be quite valuable (Thompson et al. 1993). They may be used to elucidate vegetation

changes in retrospective studies (Diekmann and Dupré 1997), explain the species composition at different N-deposition scenarios (Tyler 1987; Falkengren-Grerup and Brunet 1996) or show characteristics of species threatened by extinction (Ellenberg 1985). They can more or less accurately be used to explain the physiological responses of plants to the supply of N in laboratory experiments (Poorter et al. 1990; Fichtner and Schulze 1992). Nitrate content, nitrate reduction activity (NRA) and organic N content were positively related to the indicator values (Gebauer et al. 1988).

## 5. Concluding Remarks

The objective of this chapter was to highlight some recent advances in ecochemical research on soil – plant interactions. A comprehensive review of this entire area was certainly impossible and the authors' choice of topics to be treated might even be considered arbitrary. The authors have not paid much attention to the fragmentary but growing knowledge about the importance of interspecies interactions, including symbiotic relations, in soil – plant research.

One of the main research areas in plant ecology concerns interactions between soil chemistry and the performance or distribution of plants in natural and seminatural habitats. Basic knowledge is available from studies of cultivated plants, but it is often difficult to evaluate the ecological importance originating from physiological or agrochemical work and generalizations might sometimes give rise to erroneous conclusions.

The authors believe that future research on, e.g., acidifuge/calcifuge behaviour has to focus on the ability or inability of plants to modify their soil environment. The soil chemical 'preferences' of native vascular plants are usually apparent, often quite distinct, but control mechanisms seem to differ greatly among species, even within the same 'edaphic' category. Inherent differences in exudation of organic compounds to the soil, in nutrient uptake, as well as in the metabolism inside the tissues, are conditions controlling the 'edaphic behaviour' of plants. Superimposed on or actually interacting with these conditions are population ecology and competition dynamics. A major concern is what plants, or plants in combination with other organisms, can contribute in order to ameliorate a toxic or nutrient-poor soil environment. A problem to be elucidated is what various plant species or plants of differing soil habitats are able to do to the soil, not merely how principal or primary dissimilarities in soil properties influence the distribution and performance of plants. These considerations are valid for mineral as well as N nutritional and toxicological problems.

How native plants are able to cope with changes in ionic composition and concentrations in soils is pertinent to problems related to environ-

mental change. It is important to understand how they are able to adapt to situations which restrict their performance, e.g. when a previously N-limited system becomes limited by excess  $H^+$  or  $Al^{3+}$ , by reduced availability of phosphate, etc. Even though principally all plants, though to various degrees, might be disfavoured by such changes, the ultimate consequences on a field scale are difficult to prognosticate without thorough knowledge about the ecophysiology of individual species and the competitive interactions among plants.

What makes all forecasts particularly problematic is that short-term and long-term responses might differ greatly and even seem to be contradictory. Feedback effects which are difficult to foresee, interspecies interactions and lacking knowledge about time-scales for new soil chemical and microbial 'equilibria' to establish are constituents of this complex of research difficulties. The only way to proceed is to increase our knowledge about the quantitative importance of mechanisms, processes and reactions, in order to gain more experience and an improved understanding of inherent response characteristics of plant species to changing environmental conditions.

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## Ecophysiology of Xylem-Tapping Mistletoes

By Marianne Popp and Andreas Richter

### 1. Introduction

The term "mistletoe" has a double meaning: *sensu stricto* it applies to *Viscum album* L. (European mistletoe), but it is more generally used to describe perennial (usually woody) flowering plants which are attached to the shoot of trees or shrubs (Barlow 1987). The majority of the approximately 1300 mistletoe species fall into the two closely related families Loranthaceae and Viscaceae within the order Santalales (Calder 1983).

All mistletoes depend completely on their hosts for water and nutrients. However, they differ greatly in the extent to which they rely on the supply of reduced carbon from their hosts (in the extent of their heterotrophic carbon gain). While xylem-tapping mistletoes are capable of fixing atmospheric carbon dioxide and are therefore only partially heterotrophic, other mistletoes are parasitizing also the phloem of their hosts (e.g. several *Arceuthobium* species, *Viscum minimum*) and are regarded as 'holoparasites'. The latter group is characterized by a lack of chlorophyll and reduced (or absent) photosynthetic organs, whereas the xylem parasites are regarded as 'obligate hemiparasitic', which indicates that they cannot establish without a host, but rely only partially on host-derived carbon (Tsivion 1978). The extent to which mistletoes depend on heterotrophic carbon input from the hosts is one of the main topics in current mistletoe research and will be covered in Section 5.

The transfer of solutes between the host and the mistletoe takes place in the haustorium, a unique organ of parasitic angiosperms (Kuijt 1977). In xylem-tapping mistletoes an apoplastic continuum between the two partners of the association exists, whereas plasmodesmatal connections are absent from the haustorial interface (Kuijt 1977). Therefore, a direct symplastic transport of solutes from the host to a mistletoe does not occur. In the apoplast, solutes may be transported either along vascular (i.e. from xylem to xylem vessel) or non-vascular pathway (by contact of haustorial parenchyma cells with host xylem). The extent to which these two possible routes of apoplastic transport contribute to solute uptake by the mistletoe and its implication for active (selective) versus passive

uptake is another matter of discussion in current literature (Lamont 1983; Coetzee and Fineran 1987, 1989; Glatzel and Balasubramaniam 1987; Goldstein et al. 1989; Stewart and Press 1990).

In contrast to recent reviews which covered the whole field of parasitic angiosperms, this chapter will focus on xylem-tapping mistletoes only, trying to address some crucial questions of their ecophysiology, particularly nutrient uptake, solute accumulation and carbon gain (Press et al. 1990; Stewart and Press 1990; Seel et al. 1992; Press and Whittaker 1993; Press and Graves 1995).

## 2. Mineral Composition

Since it is evident that xylem-tapping mistletoes depend completely on their hosts for their mineral nutrition, comparisons of ash content of the two partners have been performed already in the last century (cf. Tubeuf 1923). However, even at this early stage of investigations controversy arose which organs of the two members of an association should be compared. Tubeuf (1923) pointed out that it would be more meaningful to draw comparisons between the leaves ('krautartige Teile autotropher Pflanzen') of both partners than between leaves of *Viscum album* and host branches as done in earlier studies.

However, even numerous experiments including different plant organs and determination of various elemental ratios (K/Ca, Mg/Ca, Na/Ca) did not explain why host and mistletoe tissues differ in their mineral composition to such an extent (Lamont 1983). Especially potassium was found to be highly enriched in the mistletoe leaves and active uptake by parenchyma cells at the host-mistletoe interface was suggested by Lamont and Southall (1982). Covering a whole season with measurements on *Loranthus euopaeus/Quercus petraea* association, Glatzel (1983) demonstrated that the higher foliar potassium-levels of the mistletoe are rather the consequence of the lack of retranslocation than of active uptake through the haustorial boundary layer. This finding applies to all mineral elements which are mobile in the phloem with the exception to nitrogen (see Sect. 3). Concentrations of these nutrients are kept on a rather constant level in host leaves via import in the xylem and export in the phloem. In the case of the mistletoes, the xylem-delivered nutrients are 'trapped' in the aerial parts. According to Pate (1995; Pate et al. 1991a) even essential nutrients are not retrieved from mistletoe leaves prior to abscission.

Another fact which has to be taken into consideration when relating the ion content of mistletoes to their hosts is the difference in water content of their tissues. Mature mistletoe leaves have a much higher water content per leaf area than most of their hosts (Popp 1987; Whit-

tington and Sinclair 1988; Popp et al. 1995) or, where leaf areas were not documented, dry weight to turgid weight ratio (Davidson and Pate 1992). Thus, in many cases where mistletoe ion content exceeds that of the host on a dry matter basis, the picture would be altered if data were expressed on a tissue water basis. Using the latter approach, Popp et al. (1995) showed that the same mistletoe species (*Tapinanthus oleifolius*) stored higher, lower or equal ion concentrations, depending on the host species and on leaf age. Whereas young leaves contained always higher potassium concentrations than leaves of their respective hosts (*Acacia karoo*, *Euphorbia virosa*, *Salvadora persica* and *Tamarix usneoides*), old leaves of *T. oleifolius* parasitizing on sodium-storing *S. persica* and *T. usneoides* exhibited lower potassium and much higher sodium concentrations than their hosts. The resemblance between mature mistletoes and host leaves in their cation and anion pattern confirmed the findings

**Table 1.** Na<sup>+</sup> and K<sup>+</sup> concentrations and K<sup>+</sup>/Na<sup>+</sup>-ratios in xylem saps of two *Phthirusa maritima* associations (data from Goldstein et al. 1989) and different plant parts of *Tapinanthus oleifolius*/*Tamarix usneoides* associations (data from Popp et al. 1995)

	Na <sup>+</sup>	K <sup>+</sup>	K <sup>+</sup> /Na <sup>+</sup>
Host tree	(mol m <sup>-3</sup> xylem sap)		
Mistletoe			
<i>Conocarpus erectus</i>	0.55	1.89	3.44
<i>Phthirusa maritima</i>	0.20	1.51	7.54
<i>Coccoloba uvifera</i>	1.83	2.48	1.36
<i>Phthirusa maritima</i>	0.78	2.02	2.58
Host tree/mistletoe	(mol m <sup>-3</sup> plant water)		
Plant part			
<i>Tamarix usneoides</i>			
branch, wood	300.9	107.5	0.36
branch, bark	224.4	121.9	0.54
<i>Tapinanthus oleifolius</i>			
haustorium within host-tree	318.7	134.3	0.42
haustorium bark	67.9	459.1	6.76
haustorium wood	45.2	249.2	5.51
stems, bark	175.8	462.6	2.63
stems, wood	55.0	272.6	4.96
leaves 2.0–3.0 mm	712.1	53.9	0.08
leaves 1.5–2.0 mm	407.7	48.7	0.12
leaves 1.0–1.5 mm	372.6	53.1	0.14
leaves 0.5–1.0 mm	444.6	89.2	0.20
leaves < 0.5 mm	227.1	144.1	0.63
youngest shoot tips	93.2	240.8	2.58
flowers	65.7	128.2	1.95
fruit	86.2	171.5	1.99



of Glatzel and Balasubramaniam (1987) and favours their hypothesis of a predominantly passive uptake of inorganic ions.

However, the only investigation so far comparing inorganic cations in the xylem sap of mistletoes and host suggested a potential for selective ion uptake at the haustorial interface (Goldstein et al. 1989). The higher potassium/sodium ratios of the mistletoe xylem saps are brought about by a reduction in sodium content compared with the two host species (Table 1). Selectivity is not necessarily tantamount to active transport and even for this type of comparison of xylem saps Pate (1995) stated a complication 'by possible cycling within the mistletoe'. An indication of the very different composition of the mistletoe's phloem and xylem saps comes from the detailed analysis of various plant parts of a *T. oleifolius*/*T. usneoides* association, where the potassium/sodium ratio in the phloem-fed organs like fruits, flowers and shoot tips was above unity, whereas in mature leaves K/Na ratios ranged between 0.2 and 0.08 (Popp et al. 1995; Table 1).

From the above mentioned it is evident that the existing body of evidence is not yet sufficient to decide to what extent passive and/or active ion uptake processes are involved in the mineral nutrition of mistletoes. Even if there was clear evidence for one type of association, it remains in question whether those results could be applied to other host-parasite pairs under different environmental conditions (Panvini and Eickmeier 1993; Pate 1995).

### 3. Nitrogen

#### a) Nitrogen Uptake

Nitrogen may be obtained by mistletoes either in inorganic or organic form. The presence of nitrate reductase (NR) has been demonstrated in several mistletoe species (Stewart and Orebamjo 1980; Hunter and Visser 1985; McNally and Stewart 1987), but NR activity did not show the usual correlation to total nitrogen content. This was taken as an indication that other sources than nitrate are important for nitrogen supply. The low levels of chloroplastic glutamine synthetase described by McNally and Stewart (1987) could be also related to the low potential of these mistletoe species to assimilate nitrate since the latter stages of nitrate reduction occur in the chloroplasts. On the other hand, the low activities of chloroplastic glutamine synthetase may indicate low rates of photorespiration and photosynthesis (Stewart and Press 1990).

When nitrogen content of mistletoe and host leaves are compared, in many cases the lower values were found in the parasite leaves (Orozco et al. 1990; Küppers 1992; Marshall et al. 1994b). However, again – as mentioned in Section 2 for the mineral ions – the different leaf character has

to be taken into account. Especially in warmer climates, ash content of mistletoe may account for more than 30% of dry matter and this lowers the nitrogen content calculated to that basis (Richter et al. 1995).

However, there were also mistletoe – host associations where the mistletoe partner was equal or even higher in nitrogen than the host. Into this latter group belonged a number of mimicking mistletoes, which might gain an advantage by getting less browsed by vertebrate predators when looking like their host (Ehleringer et al. 1986b). Non-mimicking species with their lower nutrient values can be easily distinguished by the consumers from the hosts.

Bannister (1989) confirmed these findings for New Zealand mistletoes also showing that cryptic mistletoes are equal or higher in nitrogen content than their hosts whereas non-cryptic species are lower. Since New Zealand mistletoes have almost certainly evolved in the absence of herbivorous mammals the hypothesis of selective grazing cannot be applied in this case. An interesting hint to explain this coincidence between nitrogen storage and leaf mimicry comes from investigations of plant hormones in xylem saps of mistletoes and hosts (Hall et al. 1987). In mimicking situations (*Amyema miquelii* on *Eucalyptus polyanthemus*), types and concentrations of cytokinins in the xylem saps of the partners were more similar than in a non-mimicking association (*Amyema pendulum* on *Eucalyptus cinerea*). In this respect – like in several others – more detailed knowledge of the processes in haustoria would be needed for a better understanding.

#### b) The 'Nitrogen-Parasitism' Hypothesis

The 'nitrogen-parasitism' hypothesis was proposed by Schulze et al. (1984) to explain that the seasonal nitrogen investments of *Loranthus europaeus* could be only matched by high transpiration rates taking into account the low nitrogen concentration of the host xylem sap (*Quercus robur*). Already at this point one may argue that in case of limiting nitrogen availability a mistletoe will rather change its growth than its transpiration according to the various models of optimizing nitrogen distribution in the canopy (Werger and Hirose 1991). Moreover, as pointed out by Press and Whittaker (1993) and Press (1995), nitrogen uptake by mistletoes is causally related to heterotrophic carbon intake, since most of the xylem sap nitrogen will be in organic form (see Sect. 5). Thus, there is at the moment no possible approach to distinguish whether transpiration in mistletoes is driven by requirements for host carbon, nitrogen and/or water.

#### 4. Photosynthesis

Comparing parameters of photosynthesis of mistletoes and their corresponding hosts reveals two consistent features: 1)  $\text{CO}_2$  net uptake is lower in the mistletoe than in its host (Hollinger 1983; Ehleringer et al. 1986a; Goldstein et al. 1989; Küppers et al. 1992; Johnson and Choinski 1993; Marshall et al. 1994a; von Willert and Popp 1995). The one exception is *Viscum laxum* on *Pinus sylvestris* (Schulze et al. 1984), where relating  $\text{CO}_2$  exchange to leaf area might have been problematic in case of the host needles; 2)  $\delta^{13}\text{C}$  values of mistletoe leaves are usually more negative than those of the corresponding hosts (de la Harpe et al. 1980; Schulze and Ehleringer 1984; Ehleringer et al. 1985, 1986a; Goldstein et al. 1989; Schulze et al. 1991; Küppers 1992; Marshall et al. 1994b; Richter et al. 1995).

The findings on the diminished photosynthetic capacity of mistletoe leaves are substantiated by results on reductions in the photosynthetic apparatus. Hill reaction activities in thylakoids isolated from *Tapinanthus vittatus* were less than half those of its host (*Diplorhynchus condylocarpon*; Johnson and Choinski 1993). Investigating the characteristics of chloroplasts isolated from a temperate (*Viscum album*) and tropical (*Tapinanthus dodoneifolius*) mistletoe species, Tuquet and Sallé (1996) found large deficiencies in photosystem activities, which correlated with low amounts of chlorophyll-protein complexes and changes in some lipid fractions. Indirect evidence for a reduced capacity of mistletoe leaves to fix carbon may be deduced from the rather low light saturation of  $\text{CO}_2$  assimilation (Orozco et al. 1990; Küppers et al. 1992; von Willert and Popp 1995) and the very low levels of chloroplastic glutamine synthetase (McNally and Stewart 1987), which indicates low rates of photorespirations and therewith photosynthesis, as already mentioned in Section 3a. In consequence, mistletoes maintained relatively high intercellular  $\text{CO}_2$  concentrations which explains the constantly more negative  $\delta^{13}\text{C}$  values (Goldstein et al. 1989; Richter et al. 1995).

#### 5. Heterotrophic Carbon Gain

As originally pointed out by Raven (1983) parasitic plants which receive nitrogen predominantly as nitrogen-containing organic compounds will also receive considerable amounts of organic carbon: assuming that 3 mol carbon are transported per mol nitrogen, he calculated that about 20% of the carbon will be derived from its hosts. It was subsequently shown that xylem-tapping root parasites, such as *Striga hermonthica*, are indeed importing substantial amounts of carbon from their host's xylem sap, beside the fact that they are fixing atmospheric  $\text{CO}_2$  at rates which

are within the range of other C<sub>3</sub> plants (Press et al 1987; Graves et al. 1989).

Marshall and Ehleringer (1990) first demonstrated a carbon flux from a host tree to a xylem-tapping mistletoe: they found that about 62% of the carbon of the leafy mistletoe *Phoradendron juniperum* was derived from its host *Juniperum osteospermum*. In this study two approaches were used: first,  $\delta^{13}\text{C}$  values of mistletoe and host leaves were determined and compared with those theoretical values predicted from gas exchange measurements (from the  $c_i/c_a$  ratio; Farquhar et al. 1982). While the measured and predicted  $\delta^{13}\text{C}$  values agreed well for the host leaves ( $-24.6\text{‰}$ ), they were different for the mistletoe leaves (predicted  $-31.0\text{‰}$ , measured  $-27.0\text{‰}$ ). Assuming that this difference is due to an import of carbon with a higher  $\delta^{13}\text{C}$  signature from the host, the heterotrophic carbon gain H was calculated to be 62% by the following equation:

$$H (\%) = (\delta_{\text{MP}} - \delta_{\text{MM}}) / (\delta_{\text{MP}} - \delta_{\text{H}}), \quad (1)$$

where  $\delta_{\text{MP}}$  is  $\delta^{13}\text{C}$  value predicted for the mistletoe,  $\delta_{\text{MM}}$  is the measured  $\delta^{13}\text{C}$  value of the mistletoe and  $\delta_{\text{H}}$  is the  $\delta^{13}\text{C}$  value for the host tissue (Press et al. 1987). This ' $\delta^{13}\text{C}$  difference method' of calculation of heterotrophic carbon gain was closely matched by the second approach of calculation in which the xylem carbon concentration of the host and the transpiration rate of the mistletoes were measured and compared with the net CO<sub>2</sub> uptake by the mistletoe ('carbon budget method').

These methods have since been used by several other authors, who all demonstrated a substantial heterotrophic carbon gain by xylem-tapping mistletoes (Table 2; Pate et al. 1991a; Schulze et al. 1991; Richter and Popp 1992; Marshall et al. 1994b; Richter et al. 1995). The 'carbon budget method' was used to estimate the heterotrophic carbon gain which ranged between 22.6 and 43% for *Viscum album* parasitizing *Malus domestica* (Richter and Popp 1992). Pate et al. (1991a) used a slightly different method: they calculated that 23.7% of the carbon of *Amyema linophyllum* was derived from host *Casuarina obesa* by multiplying the seasonal nitrogen increase with the C/N ratio of the host xylem sap. A somewhat refined  $\delta^{13}\text{C}$  approach was used to determine the heterotrophic carbon gain of *Tapinanthus oleifolius* parasitizing a CAM host, *Euphorbia virosa*, and a nitrogen-fixing C<sub>3</sub> host, *Acacia nebrownii* (Richter et al. 1995). Since it was noticed that the  $\delta^{13}\text{C}$  values for host xylem saps deviated considerably from those of host leaves (between 0.5 and 3‰ for a range of C<sub>3</sub> host trees), calculations for heterotrophic carbon gain were made with  $\delta^{13}\text{C}$  values of the host xylem saps rather than of host leaves. Hence, 56.7 and 51.4% of the carbon of *T. oleifolius* was estimated by the ' $\delta^{13}\text{C}$  method' and the 'carbon budget method', respectively, to be derived from host *A. nebrownii*. In the case of host *E. virosa*, both young and old leaves of the parasite were measured. Higher tran-

Table 2. Heterotrophic carbon gain (in percentage of total carbon gain) of xylem-tapping mistletoes

Mistletoe	Host tree	Heterotrophic carbon gain	Method of calculation	Location	Reference
<i>Phoradendron juniperinum</i>	<i>Juniperus osteosperma</i>	62%	$\delta^{13}\text{C}$ difference <sup>a</sup>	USA	Marshall and Ehleringer (1990)
		61%	Carbon budget <sup>b</sup>		
<i>Amyema linophyllum</i>	<i>Casuarina obesa</i>	23.7%	Carbon budget <sup>c</sup>	Australia	Pate et al. (1991b)
Unspecified mistletoes	<i>Aloe dichotoma</i>	67%	$\delta^{13}\text{C}$ difference <sup>d</sup>	Namibia	Schulze et al. (1991)
	<i>Euphorbia virosa</i>	47%	$\delta^{13}\text{C}$ difference	Namibia	
<i>Viscum album</i>	<i>Malus domestica</i>	23–43%	Carbon budget <sup>c</sup>	Austria	Richter and Popp (1992)
Miscellaneous mistletoes <sup>e</sup>	Miscellaneous host trees	5–21%	$\delta^{13}\text{C}$ difference	Australia	Marshall et al. (1994b)
<i>Tapinanthus oleifolius</i>	<i>Acacia nebrowii</i>	56.7%	$\delta^{13}\text{C}$ difference	Namibia	Richter et al. (1995)
	<i>Euphorbia virosa</i> (young leaves)	51.4%	Carbon budget		
		55.4%	$\delta^{13}\text{C}$ difference	Namibia	
	<i>Euphorbia virosa</i> (old leaves)	46.3%	Carbon budget		
		87.3%	$\delta^{13}\text{C}$ difference	Namibia	
		81.3%	Carbon budget		

<sup>a</sup>  $\delta^{13}\text{C}$  difference<sup>c</sup> method: the difference between  $\delta^{13}\text{C}$  value of mistletoe leaves predicted from the  $c/c_s$  ratio and the actual  $\delta^{13}\text{C}$  values for mistletoe and host leaves are used to calculate the heterotrophic carbon gain (see text for details).

<sup>b</sup> 'Carbon budget' method: the xylem carbon content and the transpiration rate of the mistletoes are used to calculate heterotrophic carbon gain (see text for details).

<sup>c</sup> Carbon intake was calculated by multiplying the annual increase in mistletoe N with the C/N ratio of the host xylem sap. The C/N ratio of the xylem sap of *Casuarina obesa* was deduced from the C/N ratio of amino acids and amides to be 5.3.

<sup>d</sup> In the absence of gas-exchange measurements, all calculations were made with  $c/c_s$  of 0.85 for mistletoes (corresponding to a  $\delta^{13}\text{C}$  of  $-31\text{‰}$ ).

<sup>e</sup> In the absence of photosynthesis measurements, calculation was made with a mean daily  $\text{CO}_2$  uptake rate of  $0.13 \text{ mol m}^{-2}$  for *Viscum album* (Schulze et al. 1984).

<sup>f</sup> The following mistletoes were sampled on various host: *Lysiana exocarpi*, *L. spathulata*, *L. subfalcata*, *Diplotia grandibracteata* and *Amyema maidonii*. However, heterotrophy data were not presented for individual mistletoe-host associations.

spiration rates and lower net photosynthesis of old leaves resulted in considerably higher heterotrophic carbon gain for the old compared with young leaves (Table 2). Other authors have estimated much lower carbon fluxes, such as 5–21% heterotrophic carbon gain for eight Australian mistletoe-host associations (Marshall et al. 1994b).

Hence, it now seems to be widely accepted that xylem-tapping mistletoes not only are water and nutrient parasites, but also derive significant amounts of organic carbon from their hosts. However, the extent to which they rely on such a heterotrophic carbon nutrition is still controversial. The above-cited studies showed that between 5 and 87.3% of the parasites' carbon may be host-derived.

#### a) How May The High Variation in Degree of Heterotrophy Be Explained?

Partly this variability in degree of heterotrophy may be due to differences between plant species (both host and mistletoe), climatic conditions (which might affect transpiration rates) and nutritional status (see Sect. 3 for discussion of nitrogen). However, some of the observed variability may be also attributed to difficulties in the methods used for determination of heterotrophic carbon gain. Accurate quantification by methods which make use of the  $\delta^{13}\text{C}$  values of host and mistletoe tissues is limited by the difference in  $\delta^{13}\text{C}$  value predicted from the internal  $\text{CO}_2$  concentration of the mistletoe and the actual  $\delta^{13}\text{C}$  of the host tissue, since small differences may yield considerable errors [see Eq. (1)]. Additionally, as already pointed out by Press et al. (1987), the quantification of carbon gain by ' $\delta^{13}\text{C}$  difference methods' is also complicated by limited knowledge of respiration, and export and import of carbon within the parasite. Another possible source of error is the assumption that the  $\delta^{13}\text{C}$  value of the carbon which is imported by the mistletoe is the same as the  $\delta^{13}\text{C}$  value of the host leaves. It was demonstrated for a range of host trees of *T. oleifolius* that the  $\delta^{13}\text{C}$  values of the xylem saps were constantly higher than those of the corresponding leaves (Richter et al. 1995). In the case of *T. oleifolius* parasitizing *A. nebrownii* the heterotrophic carbon gain would be 68.3% when calculations are made with the  $\delta^{13}\text{C}$  value for the host leaves (–23.99‰) instead of 51.4% when actually calculated with the  $\delta^{13}\text{C}$  value of the xylem sap carbon (–21.05‰). However, very little is known about  $\delta^{13}\text{C}$  values of the xylem sap and values may vary with time of day, season or climatic conditions (Leavitt and Long 1985) as well as with nutritional status. The problem of the momentary character of the xylem sap also applies to measurements of xylem carbon content and is therefore a weakness of all methods for the measurement of heterotrophy.

A further problem that applies to all measurements of carbon gain is the assumption that host-derived carbon is equally partitioned within the mistletoe, i.e. that leaves with higher transpiration rates also import more carbon from the host and that no redistribution of carbon takes place within the mistletoe itself. However, retranslocation of carbon in the phloem of xylem-tapping mistletoes has been demonstrated (e.g. Baillon 1988). In the case of *T. oleifolius* parasitizing *E. virosa* the  $\delta^{13}\text{C}$  value increased from  $-23.73\text{‰}$  in young leaves to  $-18.99\text{‰}$  in old leaves, indicating a substantial accumulation of carbon-containing compounds which originate from the host ( $\delta^{13}\text{C}$  value of  $-14.00\text{‰}$ ). However, the same mistletoe did not show any significant changes in  $\delta^{13}\text{C}$  signature with leaf age on three other host trees (*Acacia karoo*, *Salvadora persica* and *Tamarix usneoides*; Richter et al. 1995).

#### b) The Fate of Host-Derived Carbon

Host-derived carbon-containing compounds may be either accumulated in the mistletoe, further metabolized, or directly respired. One of the most important factors determining the fate of host-derived carbon therefore seems to be the type of organic compound which is transferred to the mistletoe.

Since most of the nitrogen is transported in the form of organic compounds such as amino acids and amides and in some cases also ureids a great proportion of host-derived carbon is taken up in these forms (see also Sect. 3). For example, amino acids and amides made up 85% of the total xylem carbon of several Australian tree species (*Acacia salinga*, *A. acuminata*, *Casuarina obesa*; Pate et al. 1991b). In this case organic acids only accounted for less than 15% of xylem carbon and carbohydrates were barely detectable. However, other host trees also transferred substantial amounts of carbon in the form of carbohydrates and polyols. In the case of *Malus domestica*, sorbitol (an acyclic polyol) accounted for up to 17.5% of the total xylem carbon, corresponding to about 7 mmol sorbitol-carbon/l (Richter and Popp 1992). Similar high values were reported for several cyclic polyols, such as pinitol, and for sucrose in various associations of *T. oleifolius* on a range of different host trees (Richter et al. 1995).

Sucrose and other low molecular weight carbohydrates as well as many amino acids are common constituents of both the host trees and the mistletoes. It is therefore clear that these compounds may be metabolized by the mistletoe after uptake from the host xylem sap. However, some constituents of the host xylem saps which are not indigenous in the mistletoes are obviously not metabolized after uptake, but accumulated, as it was shown for pinitol and other cyclitols (Popp 1987; Richter and Popp 1992; Richter et al. 1995), mannitol (Plouvier 1953)

and for two unusual amino acids, djencolic acid and tyramine (Pate et al. 1991b). In terms of heterotrophic carbon gain, these compounds may be regarded as useless. However, the accumulation of, for example, pinitol or mannitol could have other beneficial effects (Popp and Smirnoff 1995).

Beside those host-derived substances which are accumulated in the mistletoe, there are also major host xylem constituents, which are only found in trace amounts (or not found at all) in the corresponding mistletoes. This was shown for sorbitol, a predominant neutral compound in the xylem sap of *Crateagus monogyna*, which was barely detectable in the parasite *V. album* (Richter and Popp 1992; Wanek and Richter 1993). Interestingly, the structurally very similar compound mannitol was accumulated by the same mistletoe when growing on *Fraxinus excelsior*. That certain host-specific compounds are apparently barred access to the mistletoe has been interpreted as discriminatory processes at the haustorial interface (Pate et al. 1991b) and would suggest a predominantly selective (active) uptake. However, in the case of sorbitol it was demonstrated that metabolization of this host-specific compound took place in the mistletoe. The parasite showed relatively high activities of the enzyme sorbitol dehydrogenase, which breaks down sorbitol to fructose, when growing on sorbitol-containing host trees (Wanek and Richter 1993). Therefore, the lack of sorbitol in the parasite shoot is due to the capacity of the mistletoe to metabolize this host-specific compound rather than to a discriminatory process at the haustorium.

Nevertheless, several studies have shown that the composition of the xylem saps of hosts and mistletoes differ substantially, both in inorganic (Goldstein et al. 1989; Table 1) and organic (Pate et al. 1991b; Rennenberg et al. 1994; Richter et al. 1995; Tennakoon and Pate 1996) composition. This clearly indicates a pivotal role of the haustorium in processing host-derived nutrients. Further research in this area is therefore clearly needed.

## 6. Water Relations

There are two possible avenues for the mistletoe to attract the needed amounts of water from its host: by maintaining higher transpiration rates and/or a more negative water potential ( $\psi$ ). Higher transpiration rates in the parasites were reported for a number of different associations (Härtel 1937; Schulze et al. 1984; Ullmann et al. 1985; Ehleringer et al. 1986a, Goldstein et al. 1989); however, several detailed studies showed cases where host transpiration exceeded that of the mistletoe (Hellmuth 1971; Fisher 1983; Küppers et al. 1992; von Willert and Popp 1995). As already anticipated by Fisher (1983) the ratio between mistletoe and host transpiration depends on the water supply and the vapour pressure



deficit. *Tapinanthus oleifolius* exhibited higher transpiration rates than its host *Acacia nebrownii*, when the leaf-to-air water vapour pressure difference ( $\Delta W$ ) was beyond 30 mPa Pa<sup>-1</sup> (von Willert and Popp 1995). When more moisture was abundant, the situation was reversed, which indicates a good stomatal control in the mistletoes.

In the case of water potentials, there are only a few exceptions (Whittington and Sinclair 1988; Davidson and Pate 1992) to the rule that they are more negative in the mistletoes than in their hosts (Scholander et al. 1965; Hellmuth 1971; Fisher 1983; Glatzel 1983; Schulze et al. 1984; Ullmann et al. 1985; Ehleringer et al. 1986a; Davidson et al. 1989; Goldstein et al. 1989; von Willert and Popp 1995). This is also true for pre-dawn water potentials (Whittington and Sinclair 1988; Goldstein et al. 1989), pointing to an inability of the mistletoe to rehydrate completely during the night. The reason for this is assigned to a high resistance to the water flow through the haustorium (Glatzel 1987; Davidson et al. 1989).

The high water capacitance (Glatzel 1987; Whittington and Sinclair 1988) and the often succulent character of mistletoe leaves (Popp 1987; Whittington and Sinclair 1988; Popp et al. 1995) contribute to their ability to maintain a positive turgor at lower water potentials than their hosts. In those studies where osmotic potentials were compared in mistletoe-host associations, they were more negative in the parasites (cf. Ehleringer and Marshall 1995). However, also in this case exceptions exist (Popp 1987), which may have to do with the age of the mistletoe leaves.

## 7. Conclusion

Despite the efforts of several research groups and numerous investigations there are still many questionmarks surrounding the ecophysiology of mistletoes. One of the reasons for this is the in many cases complicated access to the plants under investigation and the difficulties of cultivation or experiments under controlled conditions. Most of the studies give just a momentary picture which provides only restricted information on flows and regulations in the parasite-host system. A main deficit in our understanding stems from the absolute lack of information about metabolic processes in the haustoria. However, they are even more problematic for taking and processing samples. Perhaps modern laser techniques or a trained woodworm will bring progress in this respect.

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